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THE BIOLOGICAL BULLETIN

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ERRATUM

THE BIOLOGICAL BULLETIN, Volume 165, Number 2, Page 514

The following correction should be made in the first abstract by A. Eisen, G. T. Reynolds, S. Wieland, and D. P. Kiehart entitled, "Calcium transients during fertilization in single sea urchin eggs," appearing on page 514. The title and authors are incorrect and the abstract should now read as follows:

Calcium transients during early development in single starfish (Asterias forbesi) *oocytes and eggs.* A. EISEN (Children's Hospital of Philadelphia) AND G. T. REYNOLDS.

Two events associated with a putative transient increase in cytoplasmic free calcium include: activation of the starfish oocyte with the maturation hormone 1-methyl adenine (1-MA), and fertilization of the starfish egg with sperm. These events were investigated in single oocytes and eggs by the detection of calcium specific luminescence from single cells injected with an acetylated form of the photoprotein aequorin (10 mg/ml in 10 m*M* HEPES, 0.2 m*M* EGTA, pH 7.0 tp 3% of cell volume). Using a microscope-photomultiplier and a microscope-image intensifier-SIT vidicon detector sensitive to $<10^{-7}$ *M* Ca^{++} we found: 1) a barely detectable ($\leq 10^{-7}$ *M*) change in free calcium from oocytes in response to 1-MA (final concentration *ca.* 150 μM), and 2) a large (*ca.* 10^{-6}) increase from eggs fertilized with sperm 15 minutes after application of 1-MA and 5 minutes after general vesicle breakdown (17°). The calcium-aequorin luminescence increases as it propagates over 30–40 s and decays uniformly over 200–300 s. The absence of a calcium transient in the *Asterias forbesi* differs significantly from the large (*ca.* 10^{-6} *M*) transient reported in the *M. glacialis* oocyte and is suggested as being a common feature of starfish oocyte activation. The calcium transient at fertilization in *Asterias* eggs is similar to that described in several species of sea urchin (*A. punctulata* and *L. variegatus*) although the propagation time is much longer in the starfish egg.

We thank Dr. O. Shimomura for the gift of acetylated aequorin. We thank Dr. A. J. Walton for the use of his microscope objectives and assistance in the experiments. This work was supported by DOE Contract EY-76-S-02-3120 to G.T.R.

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TISSUE GRAFTING IN ANIMALS: ITS DISCOVERY IN 1742 BY ABRAHAM TREMBLEY AS HE EXPERIMENTED WITH HYDRA

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(*Leslie Wolcott, Artist*)

Department of Developmental and Cell Biology, University of California, Irvine, California 92717

INTRODUCTION

Abraham Trembley of Geneva (1710–1784), while employed as a tutor-in-residence for the two children of Count Bentinck of the Hague in Holland, rocked the scientific world with his discoveries concerning the "Polyp with arms shaped like horns," later designated as the genus *Hydra* by Linnaeus in 1746. In addition to carrying out the first grafts ever made using animal tissue, Trembley, while using hydra, made a number of other major biological discoveries (see Baker, 1952). During four productive years, Trembley proceeded to: (a) perform the first rigorous experiments on regeneration in animals; (b) prove that asexual reproduction can take place in animals by budding; (c) demonstrate that tissues can be vitally stained and that these stained tissues can be used in experiments; (d) describe properties of materials which fit Dujardin's description of protoplasm reported almost 100 years later in 1835; and (e) show that "eyeless" animals can respond to light.

Although the technique of grafting animal tissues together is widely used in experimental biology and in medicine, few scientists know how and where the technique originated. Interestingly, among those who rightfully credit Abraham Trembley of Geneva with carrying out the first animal grafts on the freshwater hydra (Trembley, 1744), some report incorrectly how the very first of these grafts were made (*e.g.*, Baker, 1953). In this review we describe the first animal grafts as presented by Trembley in his *Mémoires* (1744), and show how they originated from his experiments on turning hydra inside out. Where appropriate, we use Trembley's own words in direct translation or in paraphrase, together with copies of the original illustrations published in 1744.

By describing the details of his experiments and the thoughts behind them, we hope to introduce many biologists, who are but dimly aware of Trembley, to a remarkable figure in the history of biology. Unlike most of his peers, Trembley conducted and reported his experiments with a detail, caution, logic, and rigor rare for his time. In recognition of his astounding discoveries, he was elected to the Royal Society of London and in 1743 was awarded its prestigious Copley Medal, considered then to be one of science's highest honors.

Finally, although virtually all the observations and experiments reported in Trembley's *Mémoires* (1744) have been confirmed over the past two centuries and still serve as models for much research in experimental morphology and developmental biology, the processes taking place at the cell and tissue levels that underly many of his observations remain to be elucidated. Some of these unanswered questions become apparent as we discuss the classic grafting experiments Trembley performed in the fall of 1742.

Received 14 November 1983; accepted 28 November 1983.

TREMBLEY'S MAJOR EXPERIMENTAL ANIMAL, THE FRESHWATER HYDRA

In hindsight, hydra, a diploblastic animal with no organs, proved to be ideal for grafting experiments. It is simply constructed, shaped like a two-layered tube with a "foot" on one end with which it adheres to surfaces, and a mouth at the other end surrounded by a ringlet of tentacles. The cell layers are epithelial in nature, consisting of an ectoderm on the outside and an endoderm on the inside. Both are attached to a primitive basement membrane known as the mesoglea or mesolamella. Finally, there is little chance of pieces of hydra from different animals of a colony being significantly different in their genetic makeup from each other because each member of the colony usually originates asexually from a single individual by budding.

REGENERATION AND WOUND HEALING, AND THE FIRST ANIMAL GRAFTS

Trembley's discovery that separated cut pieces of hydra could regenerate, and his observations of the ensuing wound healing taking place in those separated regenerating pieces, most likely prepared him to accept the findings that he would subsequently observe while making animal grafts.

For example, he best describes wound healing while observing the rejoining of the cut edges of strips of hydra cut longitudinally from the body column. Trembley writes that most frequently each half first twists itself about and curls up forming a coil. "Then the coil straightens out; and, after a while, the edges of the two sides draw together and join, forming a tube. . . . In reuniting, these edges join so well that from the very first moment no scar can be seen at the place where they come together. The skin of the Polyp is as smooth there as it is elsewhere."

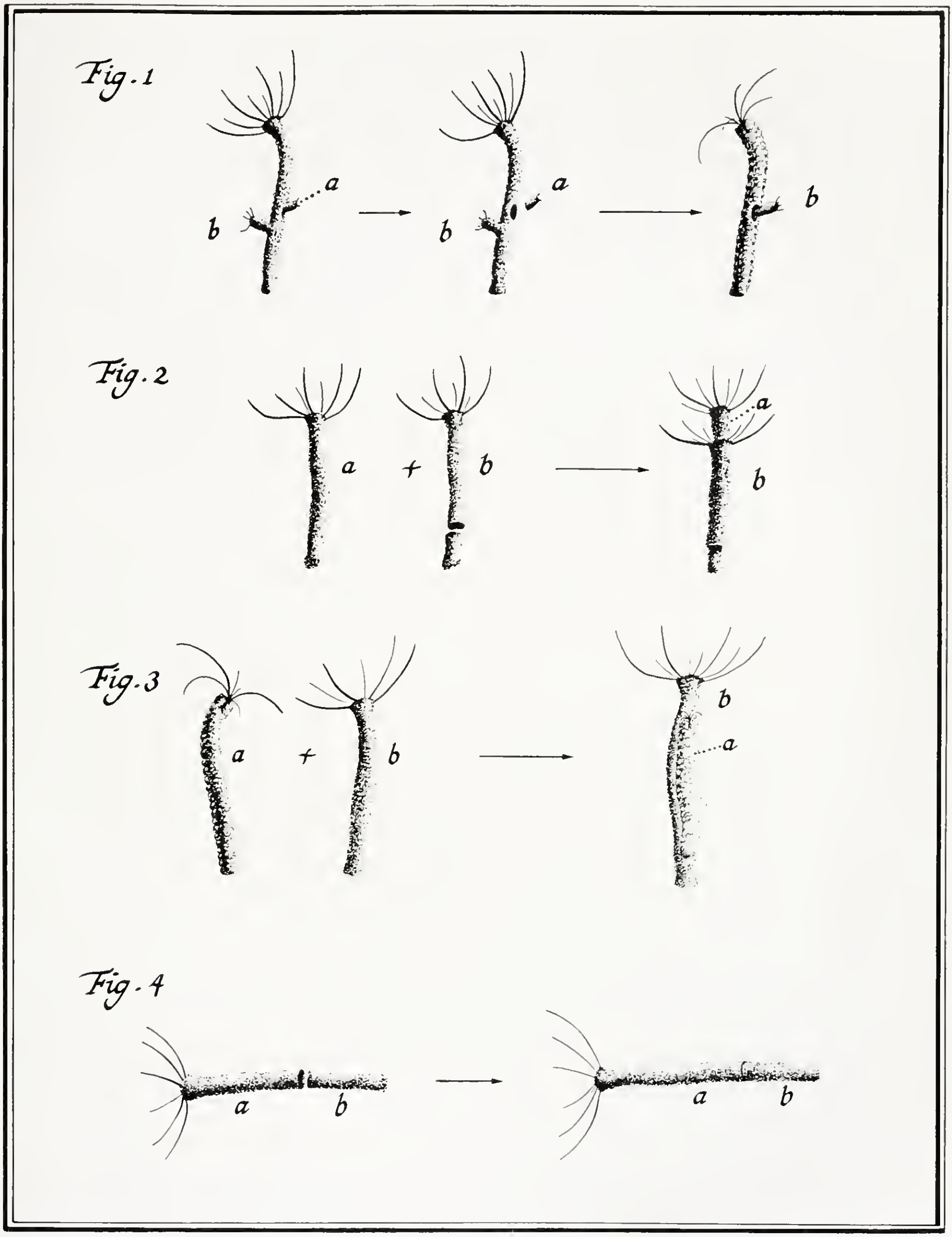
GRAFTING

After describing the rejoining of cut edges of the same piece of tissue and general experiments on creating animals like the "many headed hydra" (Trembley's first use of the term "hydra"), he proceeds to his experiments on turning hydra inside out. These experiments provided Trembley with the first clues that tissues from separate polyps can graft together. They stimulated him to embark on a series of logical experiments which eventually provided conclusive evidence that animal tissues can graft together.

First, we outline the design and order of experiments related to grafting that Trembley reports; we describe each in detail in the ensuing pages: (a) While observing a hydra that he had turned inside out, Trembley noted that a bud became permanently grafted to a cut opening in the parental tissue (Fig. 1). (b) Next, in order to prove that two separate hydra could also join in a like manner, he inserted a whole hydra into another hydra and found that the two joined permanently (Fig. 2). (c) Intrigued with this finding, he tried an unusual variation of inserting an "inside-out" hydra into one that was not (Fig. 3) and he found they "joined" into one. (d) Trembley lined up cut portions from two separate hydra next to each other, and they joined (Fig. 4). (e) Following those successful experiments, he tried to determine if portions of hydra of different species would graft together. (f) Finally, through letters, Trembley informed other scientists of his findings, and in the *Mémoires* he reports their verification of his discovery of tissue grafting in animals.

(a) Bud to parent grafts from hydra turned inside out

We find Trembley's original rationale for turning hydra inside out interesting, because through such an undertaking he had hoped to achieve the very first goal that



FIGURES 1–4: Experimental procedures leading to successful grafts between portions of hydra (Artist: Leslie Wolcott).

FIGURE 1. Mature bud grafting to a cut opening in inverted parental body column. See text for explanation. Hydra at right is inside out except for the bud protruding through the cut opening.

FIGURE 2. An intact hydra is inserted into another one that has had its foot removed. See text.

FIGURE 3. Inserting an inverted hydra (left) into one that was not inverted (center). See text.

FIGURE 4. Severed portions of the same or different hydra are lined up with cut ends touching each other. See text.

H. M. Lenhoff set out to accomplish under the tutelage of W. F. Loomis more than 200 years later—to grow hydra on a liquid medium ("nutritive juices"). Such an effort is usually the first of any present-day biologist developing a new experimental organism for research. Trembley wrote, "Since I was unsuccessful in finding a substance in which I could place the Polyps and which would serve to nourish them, I thought of inverting them so that the external surface of their skins would form the walls of their stomachs. I had very little confidence that I would see this experiment succeed, but I did not think it proper not to try it."

It was a complication of turning hydra inside out that led Trembley to observe his first graft. Recall that hydra are constructed like simple tubes, and, as might be expected, Trembley found that he could turn such tubular, nonbudding animals inside out with relative ease. What happened, however, when the hydra he was inverting had one or more immature buds that were still connected by a tubular opening to the parent? "After the operation, the young end up inside the parent's body . . . [In the case of these immature buds,] the connecting passage between the stomach of the mother and that of her young remains undiminished in size. When the mother is inverted, the offspring is able to invert itself and does. It is precisely as though the fingers of an inverted glove were able to invert themselves unaided. . . ."

Trembley prepares us for his momentous discovery when he writes, "I believe that the following observations made on a partly everted Polyp are worth bringing to your attention." Before inverting a hydra on which two immature buds had begun to sprout, he cut off one bud close to the body column of the parent animal, leaving a hole in its place (Fig. 1). The bud he chose not to sever continued to mature and was about ready to detach when Trembley inverted its mother. "I saw clearly that after the inversion the young Polyp situated inside the mother's stomach had emerged at the opening in the mother's skin made by cutting off the other offspring." Later it appeared that the young polyp had attached itself to the cut opening in the hole in the mother's skin and "seemed completely united to it," that is "the young one had grafted itself" to the inverted parent. Trembley concluded, "This Polyp was certainly most unique, composed as it was of an offspring and a portion of its mother onto which it was grafted." The question remained, Trembley continued, whether the young polyp would remain united to the mother or whether a constriction would form at the junction and the two portions would eventually separate. A constriction did not form, the portions did not separate, and Trembley thus ended his description of the first experimental observation of animal tissues grafting to each other, in this case a bud to an opening cut in the skin of its mother. Although we question some of Trembley's interpretations of the mechanisms of that accidental graft and of the fate of the two epithelial layers, there is no doubt that the graft took and was a permanent one.

Some may ask if this example is really a case of grafting two individuals. After all, couldn't one argue that a bud is truly part of the parental tissue? Yes—and no. One of the advantages of using hydra as a laboratory animal is that all hydra in a new clone are genetically alike, having descended asexually from a single individual by budding. Hence, a new bud is no different genetically from its parent (except for an occasional somatic mutation that might have taken place) than a descendent many generations removed. It makes no difference, therefore, whether one grafts a recently detached bud to its parent, or if the graft is made from a hydra taken from the same clone a number of generations removed from that parent. Both graft equally well to the parent, which was the initial cellular source of the stock, or, for that matter, to any member of that clone or species.

(b) Grafts between one hydra inserted within another hydra

The stimulus for grafting two different hydra together also came from his experiments with inverting hydra. "While observing Polyps that were partially everted, I often wondered what would become of the inverted portion of the Polyp that was covered by the everted portion. It occurred to me that perhaps, when the surfaces of the two portions were set against each other, they would fuse so that henceforth they would form but a single skin." Trembley then conjectured that if such were the case, a polyp could perhaps fuse with another polyp that was placed inside its stomach to form a single animal.

Stimulated by this conjecture, Trembley proceeded to design an orderly set of experiments which, although they did not give the single polyp he hypothesized, did conclusively prove that two separate hydra can permanently graft to each other.

To set up his experiment, Trembley placed a boar's bristle in the mouth of the polyp that was to be inserted, and thrust it to the furthest end of its stomach. Before inserting that hydra into another one, he cut off the tail end of the external host animal, and immediately "spitted" them together by inserting the boar bristle and its impaled hydra through the mouth and the end of the cut hydra. The resultant association had the head of the inserted hydra emanating a slight distance through and above the mouth of the external cut hydra (Figs. 2, 5). Likewise, the "foot" of the inserted hydra protruded a short distance from the end of the cut portion of the external animal (Figs. 2, 5). Before removing the bristle, he used another one to hold the two animals together by piercing both of them through their sides approximately in the middle of their body columns.

Although we have summarized in one paragraph the procedures Trembley designed, they were not simple to perform. Trembley held the hydra in a drop of water in the palm of his left hand while he carried out the operation, manipulating a fine scissor or a boar's bristle with his right hand. At most, he was aided by a magnifying glass.

This "insertion" experiment did not always give the same result. In some cases, the inner polyp would slowly tear through the outer one, beginning the tear at the bottom cut edge and ending at the lips, eventually healing so that Trembley would end up with two separate, perfectly formed animals. But it was his third insertion experiment that gave an "outcome . . . very different from the previous two," and that gave Trembley his first solid clue that grafts can form between two separate individual animals of the same species. Because this experiment is so elegant, we describe it in Trembley's own words and with his illustrations. Recall that he begins with one hydra inserted inside a second which has had its tail end cut off (Figs. 2, 5).

"These two Polyps had not altered their position by the 12th of October, on which day I drew out the bristle that held them pierced together. That evening, however, I noticed that the extremity *c a* [. . . Figure 5] of the inner Polyp, which was protruding from the outer Polyp's mouth *a,* was much longer than on the preceding days. I do not know whether that was the case because the extremity *c a* had elongated more, or because the inner Polyp was in fact emerging further out of the outer Polyp's mouth. On the 13th day of October, the tail portion of the inner Polyp tore through the outer Polyp's body from the bottom upward to the area marked *i* [Figure 6]. The portion *i d* marks the tail of the inner Polyp which has broken through the outer one. On the 16th, they remained as they are shown in Figure [6].

"I fed a worm to the inner Polyp *c a i d,* the only one of the two Polyps which could eat because the outer Polyp's mouth *a* was filled with the part of the inner

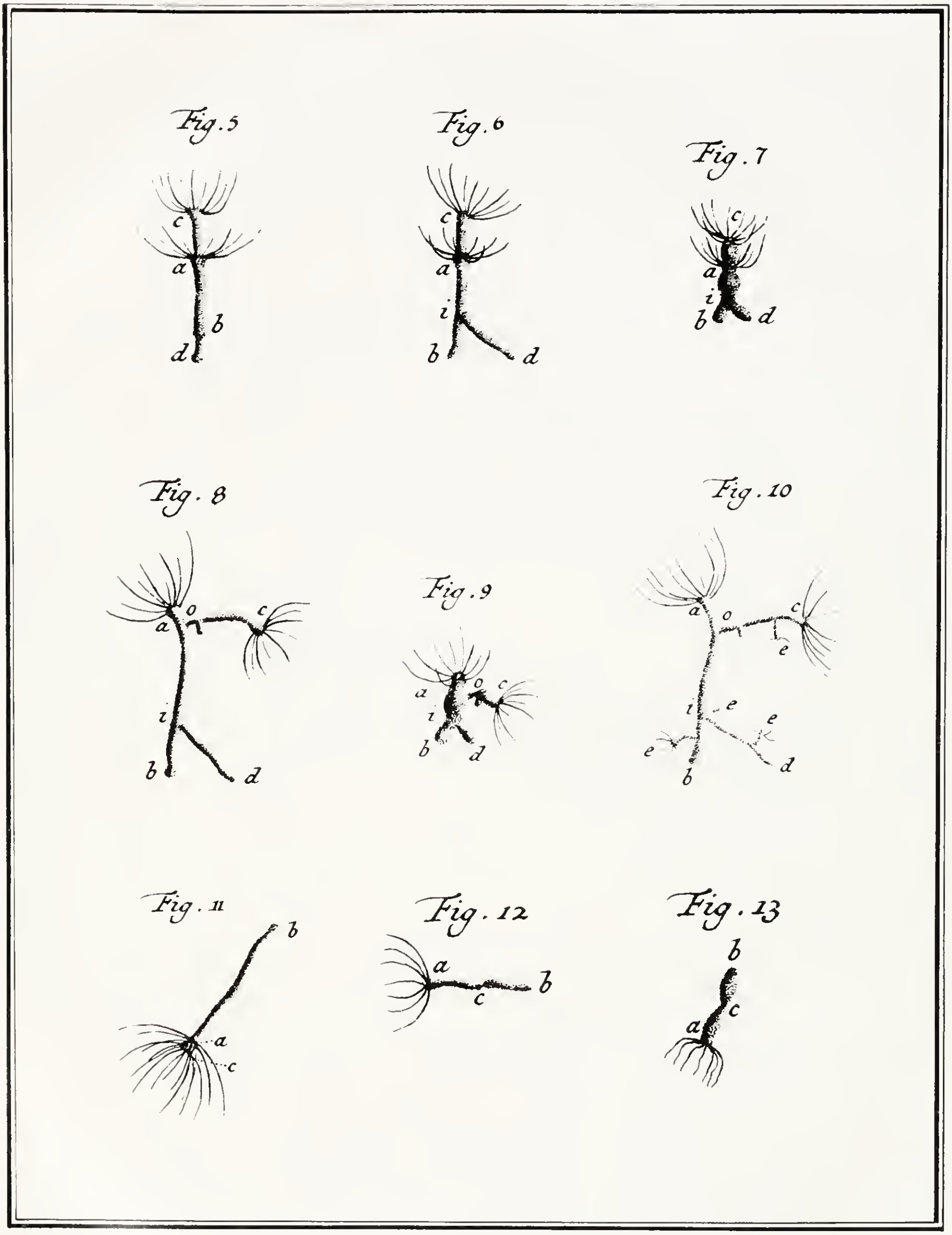


Fig. 5
c
a
b
d
Fig. 6
c
a
i
b
d
Fig. 7
c
a
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Fig. 8
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Fig. 11
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Fig. 12
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Fig. 13
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a

FIGURES 5–13: Drawings from Trembley's original *Mémoires* illustrating various stages of some of his grafting experiments. The drawings, made by P. Lyonet, have been renumbered and rearranged to fit the text of this article.

FIGURE 5. Insertion of one hydra within another one that has had its foot end cut off.
FIGURE 6. The same hydra from Figure 5 after they had grafted to one another.
FIGURE 7. The same graft from Figure 6 a few hours after mouth, *c*, had ingested food.
FIGURE 8. The same graft three days later.
FIGURE 9. The same graft a few hours after mouth, *a*, had ingested food.
FIGURE 10. The same graft showing new buds, *e*, developing at many sites.

Polyp that was emerging from it. I noticed, however, that the food spread not only through the inner Polyp which had eaten it but also through the outer. This fact was easily confirmed by the swelling of the posterior end *i b* [Figure 7] of the outer Polyp *a i b.* If the food had spread only through the inner Polyp *c a i d,* then only the portions marked *c a, a i* and *i d* would have swollen. The portion *a i,* because it was a part of the outer Polyp *a i b,* would have appeared inflated only because it contained the portion *a i* of the inner Polyp hidden underneath it. Figure [7] shows the Polyps as they appeared some time after the inner Polyp had eaten. It is easy to observe that the portion *i b* which forms the outer Polyp's posterior end has swollen and has filled with food. In order for the food to have passed from the inner to the outer Polyp, the portion *a i* of the inner Polyp [. . . Figures 6 and 7] must have been open somewhere.

"By the 19th of October, the anterior end [Figure 6 *c a*] of the inner Polyp had changed its position very markedly. On that day it no longer emerged from the outer Polyp's mouth at *a,* but, having torn through the lips of the outer Polyp, it protruded from its body a little below the head. This new configuration is shown in Figure [8], where the letters *c o* designate the anterior end of the inner Polyp, marked *c a* in Figure [6].

"Since the mouth of the outer Polyp [Figure 8*a*] was now cleared, I attempted to feed it a worm. The Polyp swallowed it and after the worm had been digested, its nutrient juices spread perceptibly throughout all the parts of both Polyps [Figure 9 *a i, c o, i b,* and *i d*]. I saw the same thing happen on the 22nd after I had fed a worm to the portion *c o;* the food also passed from this portion into the other sections of the Polyp. Thus, there was not any reason to doubt that the inner Polyp, comprised partly of sections *c o* and *i d* [Figure 8], had formed a connecting passage to the outer Polyp *a i b,* that is to say that the inner Polyp was open between *i* and *o.* It is impossible for me to describe the condition of the portion *a i* of the inner Polyp concealed within the outer shortly after the opening developed. But I can attest that it subsequently merged with the portion *a i* of the outer Polyp, without presuming to explain, however, how this [process] occurred.

"For about three months I continued to examine the portion *a i* from many perspectives; and it always appeared to me like a portion of an ordinary Polyp. The portions *c o* and *i d* of the inner Polyp had become as if grafted onto the outer Polyp *a i b.* They were connected to it as are young Polyps with their mother. These two united Polyps grew and multiplied as indicated in Figure [10] which shows them as they were the 6th of November. At that time they were sprouting four offspring *e, e, e, e,* and a few young had already detached from them. The anterior portion *c o* of the inner Polyp seemed farther away from the head of the outer Polyp than it had been on October 19th [Figure 8]. I had every reason to believe that this difference was not the result of its changing its place, but rather the result of an elongation of the portion *a o* [Figure 10] of the outer Polyp. . . .

"This last experiment that I have just reported indicates that one Polyp in a way can be grafted on to another. It has shown us at the least that the portions *c o* and *i d* [Figure 10] of the inner Polyp remained united for a long time to the outer Polyp *a i b,* as a bough or a slip is united to the stock to which it has been fastened."

FIGURE 11. The result of inserting an inverted hydra into an uninverted one (see Fig. 3).

FIGURE 12. A severed anterior portion and a severed hind portion of hydra *a b* placed together at their cut edges, *c.*

FIGURE 13. The hydra that grafted together was from the portions illustrated in Figure 12, shown here a few hours after the head end, *a,* had ingested some food.

(c) Fusing two hydra into one

The experiment to which Trembley next proceeded is one of his most extraordinary. In short, he hypothesized that if one inserted an inverted hydra inside an uninverted one, the two would fuse, and thereby form one animal (Fig. 3). It was this experiment, in fact, which gave Trembley the results he had expected to find in the previous one. Again, Trembley's words:

"This [possibility of grafting] was not the only question I was pursuing when I started to insert Polyps into each other. I wanted to see whether the inner Polyp would fuse with the outer without emerging from it. Would the inner act as a sort of lining, the two Polyps forming a single animal?

"Of all the experiments I performed with this goal in mind, none fulfilled my expectations better than the one I started on October 22nd, 1742. The Polyp which I inserted into another on that day never emerged from it, either in whole or in part. I had inverted the Polyp before inserting it; and, as usual, I carefully spitted the two Polyps together after one was inserted inside the other. I cannot explain what became of the body of the inner Polyp, whether it was dissolved in the outer Polyp's stomach, or whether the inner body merged with the outer. I can definitely state, however, that I could still see the inner Polyp's body lying inside the outer Polyp several days after it had been inserted. As for the inner Polyp's head, I was certain that it had fused with that of the outer Polyp. The lips of the outer Polyp adhered to the inner Polyp's neck, and after a certain time the two heads of these Polyps formed but one which had two rows of arms. . . . [Figure 11 *a* and *c*]. On various occasions, while feeding the Polyp *c a b* [Figure 11], I saw clearly that it had but one mouth, the one belonging to the inner Polyp. The mouth of the outer Polyp [indicated at *a*] was filled by the inner Polyp's head; or, rather, it no longer was a mouth. Only the arms which had lined its lips could be distinguished. They formed the second row of arms [Figure 11 *a*] that I just mentioned.

"I raised this Polyp from the 22nd of October 1742 until the middle of February, 1743 when it died of sickness. During this time it grew and multiplied, and I was always able to distinguish the two rows of arms on its head.

"This specimen . . . [Figure 11] belongs among the more extraordinary Polyps that we have been discussing till now. It was composed of an outer and an inner Polyp or, at least, of the anterior portion of the inner Polyp which could easily have become a complete Polyp on its own. Thus it is certain that it was composed of two Polyps and that being the case, this experiment can be viewed as the opposite of the first one we performed on the Polyps. The first [on regeneration] taught us that from one Polyp two could be made; and this one teaches that two Polyps can be made to form one."

Undoubtedly this is one of Trembley's experiments that needs to be repeated (Ischikawa, 1890, claims to have done so) and investigated more rigorously. Many fundamental questions remain to be answered, such as the mechanism of fusing and the fate of the ectoderm of the inverted hydra. Papenfuss (1934) has confirmed, for example, that the endoderm layers from two separate hydra of the same species rapidly fuse together. From Trembley's viewpoint, the results of his experiment gave him much satisfaction in that he showed that two complete separate hydra can "fuse" to form one normal-sized animal. In subsequent experiments, he also was able by conventional means to link pieces from two separate animals to form a full-sized hydra (Fig. 4).

(d) Linking severed portions of one or more polyps together

Trembley's methods of grafting, ingenious as they were, seem tedious and impractical to those of us who use hydra today in grafting experiments in our research in developmental biology. Usually we unite different portions of cut hydra by placing the cut edges next to each other. Trembley, too, used such a method after discovering "accidently," as he wrote, that "two pieces of Polyp which I had used in an experiment . . . were clinging together."

This chance observation prompted Trembley to verify experimentally this end-to-end grafting with his customary care (Fig. 4). He placed the two halves of a sectioned polyp in a dish containing a few milliliters of water, lined up the portions so that the cut ends touched (Fig. 12), and made certain that they lay touching while he observed the undisturbed polyps through a magnifying glass.

Trembley reported that such cut portions usually unite (Fig. 13) and that "the larger the edges coming into contact, the more probable it is that they will join. Such, at least, is my conjecture. . . . At first the two parts unite only through a portion of their [cut] extremities. The Polyp they form is tightly constricted at the place where the joining has occurred. As the Polyp gradually grows, however, the constriction lessens and finally disappears entirely."

Each of these experiments was done with pieces taken from a single animal. Trembley then proceeded to unite pieces taken from two separate hydra, both of the same species. First he did the since oft-repeated experiment of uniting the top half of one animal and bottom half of another and *vice versa.* In one of these experiments it happened that one of the original animals was paler in color than the other one, and this difference in coloration served as a useful vital marker of the two portions of the graft. After he had fed the reconstituted grafted animal a number of worms over a period of a few weeks, he found both that the constriction between the grafted halves disappeared, and that the entire animal had the same pigmentation. (NOTE: Earlier Trembley had experimented with staining hydra different colors by feeding them food of different pigmentation, such as red *Daphnia,* or pieces of black flatworms.)

(e) Grafts between pieces from two different species of hydra

Today we generally agree that it is impossible to make permanent grafts between pieces of hydra taken from specimens of two different species. Trembley first described this species tissue incompatibility with his typical clarity, caution, and humility. "I have also attempted to join together parts of Polyps of different species. I cannot say that I succeeded. I saw only two pieces, one from the second [species], and one from the third, which remained slightly attached for fifteen days before separating again. However, I have not repeated this experiment often enough nor with sufficient care to assert that it cannot succeed."

(f) Verification

As was customary with Trembley, he related the results of his experiments on the polyps to his friend and idol, the prestigious French scientist Réaumur. Their correspondence was published two centuries later by Trembley's great grandson, Maurice (1943). An excerpt of a letter from Réaumur dated December 14, 1743, is given in Trembley's fourth *Mémoire.* In the letter Réaumur informs Trembley that he had accidently observed some polyps cut in two which united after he had "cast them one on top of the other into an extremely conical vessel." Réaumur related how he then stimulated a Doctor de Villars to try to graft pieces of a sea anemone together, and how de Villars had succeeded in a few instances.

Trembley was elated with Réaumur's letter, for not only did its contents confirm his findings, but also the observation on the sea anemone served as further evidence that the remarkable properties encountered in his polyps were also to be found in other animals.

CONCLUDING REMARKS

In this short review we have tried to present but a few of the discoveries of Abraham Trembley that were outstanding for their time, focusing primarily on his experiments which demonstrated for the first time that animal tissues can graft together.

In particular, we have emphasized the rationale behind Trembley's experiments, the doubts that he had while carrying them out, and the details of his procedures. To our minds, his humility and respect for experimental detail are virtues still worthy of emulating. That Trembley was a pragmatic and operational biologist at a time when most biologists were prone to philosophical and pedagogical generalizing and speculating is certainly remarkable. Ironically, these virtues most likely account for his relative obscurity in the current literature on the history of biology. This article is the second (see also Lenhoff, 1980) of a series in which we attempt, on the two hundredth anniversary of Trembley's death, to reintroduce this important experimentalist to today's biologists.

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THE INFLUENCE OF THE ORIENTATION, ROUGHNESS, AND WETTABILITY OF SOLID SURFACES ON THE BEHAVIOR AND ATTACHMENT OF PLANULAE OF *CYANEA* (CNIDARIA: SCYPHOZOA)

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ABSTRACT

In the laboratory the planulae of *Cyanea* are exposed to a choice of substrates. Their behavior, the length of time required for them to attach, and the size they attain after attachment differs between glass (hydrophilic) and plastic (hydrophobic) surfaces. The correlation of these differences with wettability is shown by giving planulae a choice of six substrates exhibiting different degrees of wettability (determined by measurement of contact angles); planular attachment to these surfaces is directly proportional to the hydrophobicity of the substrate for those with contact angles from 17° to 82°, but their response is variable and relatively weak to the more hydrophilic surfaces. The same relationship between planular settlement and surface wettability is possibly shown by the preferred attachment of scyphozoan planulae (three species) to roughened plastic surfaces paralleling the increased hydrophobicity of this surface that roughening effects. However, a favorable surface (plastic) does not supercede the requirement that it also must be horizontally oriented to permit attachment to its lowermost surface; planulae rarely attach to uppermost surfaces, nor to vertically oriented surfaces.

Planulae exposed to natural substrate do not usually attach to clean shells in the laboratory nor are they found attached to fresh shells in the field, both of which have wettabilities equivalent to or less than that of glass. Aged shells, on the other hand, possessing organic and bacterial films, show increased hydrophobicity, and have a high incidence of planular attachment in the field.

INTRODUCTION

Free-living larvae characteristically attach to substrate suitable for the survival of subsequent benthic phases of their life history. This phenomenon is documented for numerous marine larvae (Meadows and Campbell, 1972; Crisp, 1974; Scheltema, 1974; Chia and Rice, 1978). Many of these investigations focus on the behavior of larvae in response to physical features of their environment (levels of illumination, temperature, salinity, current speed, substrate texture) or to specific biological substrates, such as algae on which the attached stage of a particular species is usually found; algal extracts applied as films to otherwise unfavorable surfaces induce these larvae to settle (*e.g.*, Williams, 1964; Nishihira, 1968; Ryland, 1976). Other organic films (Crisp and Meadows, 1963) and bacterial populations (Meadows and Williams, 1963; Müller, 1969; Cameron and Hinegardner, 1974; Brancato and Woollacott, 1982; Kirchman *et al.*, 1982a, b) influence the extent of larval attachment, though the presence of a bacterial film does not necessarily elicit the same response even among closely similar larval types (Crisp and Ryland, 1960; Ryland, 1974, 1976).

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Mihm *et al.* (1981) show how different combinations of contrasting substrate with organic films, with or without bacteria present, effect the wettability of these surfaces and the subsequent variable response of larvae to them. This physical characteristic of surfaces, their wettability, even of clean surfaces free of contaminating films, also affects adherence of marine larvae (Eiben, 1976; Brewer, 1979; Mihm *et al.,* 1981). This demonstration of the interaction among larvae, water molecules, and surface characteristics clarifies otherwise inexplicable results when larvae are presented different material in the laboratory, and adds another dimension to be considered in the analysis of larval distribution in the field. The specific processes of attachment on suitable substrate to which motile larvae are compelled by their behavioral reflexes involve subtle mechanisms.

The planula of *Cyanea* shows behavioral responses bringing it into appropriate contact with substrate interpreted to increase the likelihood for survival of the polyp stage into which it develops following its initial encystment (Brewer, 1976a, b). The planula is a stereogastrula (Mergner, 1971; Campbell, 1974) possessing a slightly differentiated ciliated ectoderm surrounding a simple endoderm (*e.g.,* Widersten, 1978; Martin and Chia, 1982). It lacks complex sensory organs characteristic of the larvae of higher Metazoa, yet behaves with apparent equal effectiveness in selecting suitable substrate upon which to settle (Brewer, 1976a). The interaction with a surface and its immediate environment shown by planulae also parallels this response shown by the larvae of higher forms (Chia and Bickell, 1978), contributing both to a clearer interpretation of laboratory results and to a more complete understanding of their natural distribution in the field.

Planulae of *Cyanea* are exposed to different materials in various combinations to determine their behavior in contact with, and their attachment to, clean surfaces in the laboratory. Glass and plastic coverslips are the substrate used to ascertain the relationship between behavior and attachment of planulae, the effect that substrate orientation and texture has on their attachment, and the temporal sequence during which choice of these substrates is made. The frequency of attachment of planulae to these and to other inorganic substrates exhibiting a wide range of wettability is determined and compared to the wettability of natural substrate (shell) on which the attached stages of *Cyanea* frequently occur in the field.

Materials and Methods

The experiments were done at 19 ± 1.6°C using filtered natural sea water (28‰) collected from the site from which the medusae bearing planulae were collected, and were exposed to fluorescent illumination approximating a diel cycle except during observations requiring twenty-four hours to complete. The planulae, borne on the oral folds of medusae, were obtained the same day as the experiments were set up. The coverslips (Arthur H. Thomas Co., Pennsylvania) of plastic and glass used as artificial substrate were supported about 3 mm above the bottom of their experimental containers by glass beads fastened to their corners with silicone aquarium cement, and were soaked in sea water for at least twenty-four hours, after which time they were thoroughly rinsed before planulae were exposed to them. Roughened substrate was prepared by rubbing the surface of plastic coverslips with sandpaper (100 grit).

Control observations were made, prior to carrying out the experiments described below, to determine the relative frequency of encounters by planulae with plastic or glass coverslips. Planulae were introduced into a 10 cm diameter bowl containing three coverslips of each type. The number of planulae encountering each coverslip during a one minute observation period within a microscope field at 25× magnification (8.5 mm diameter) was recorded seven times at two hour intervals from between two to ten hours after their introduction (n = 21 for each coverslip type).

The behavior of planulae after contact is made with a substrate, compared both with respect to upper and under surfaces as well as to coverslip material, was recorded for 20 planulae (10 upper surface; 10 under surface) observed for three minutes on each substrate, and for 10 planulae on the under surface of each of 10 coverslips (5 plastic; 5 glass) where their behavior upon contact was noted and timed beginning eight hours after their introduction into the bowl (n = 50 for each coverslip type).

The attachment of planulae to plastic coverslips held at two different angles were compared for eight horizontally oriented coverslips and for eight vertically oriented coverslips; four coverslips at each orientation were roughened on both sides. The vertical coverslips, held at 90° by paired glass beads fastened on opposite sides of each of the two lower corners, had their lower edge in contact with the dish bottom so that planulae could not swim beneath them. The four treatments were arranged in a Latin square in an 18 cm diameter bowl. The planulae were allowed to attach (36 hours) and the numbers on the upper and under surfaces (horizontal) and on either side (vertical) were counted.

The temporal sequence of attachment by planulae to plastic and to glass substrates, and to other surfaces in a 10 cm diameter bowl was determined by counting planulae attaching to three plastic and to three glass coverslips (upper plus under surfaces) at hourly intervals for twenty-four hours (except for hours 9, 16, and 18). The time that planulae began to attach to the hyponeuston, to the dish bottom beneath the coverslips, and to the upper surface of coverslips was also noted.

The relationship between attachment and the wettability of surfaces was found by determining the contact angle (measured with a goniometer eyepiece) formed by a 0.03 ml drop of distilled water on surfaces to which planulae were exposed; the contact angle measured is the same as that used by Eiben (1976) and Baier *et al.* (1968) based upon the description of Young (1805), except where otherwise noted, below. Five measurements at 20× magnification were made for each substrate between ten and ninety seconds after the drop was deposited; the temperature was 21°C and the relative humidity, 75%. Four treatments (coverslips of glass and of plastic, and each of these materials coated with siliclad) were arranged in a 22 cm × 34 cm glass dish as a modified 8 × 8 Latin square giving 16 replicates for each treatment, and three treatments (glass coverslips polished with commercial lens cleaner, opal, and mica) each with 4 replicates were symmetrically arranged within this pattern. The total number of planulae attaching to each of these substrates was counted sixty hours after they were placed in the dish. The diameter of 50 attached planulae (planulocysts) on each coverslip (except those treated with lens cleaner) was measured to the nearest 0.5 graticule unit (1 graticule unit = 18.2 μm) at 50× magnification using reflected illumination. These measurements were made on "blind samples," coded by an assistant, and in haphazard order relative to the treatments they may have represented. The contact angle formed on roughened coverslips and on several organic substrates, scallop and wave-worn pieces of other bivalve shells, was also determined. The scallop shells were obtained by dredging and were divided into three categories: fresh (removed from live individuals), non-eroded (lightly filmed and bearing newly settled barnacles), and eroded (lightly filmed exhibiting a bronzy iridescence and bearing planulocysts). Ten measurements of contact angles made by an air bubble on the inner surface of submerged scallop shells in each category were made (after the method of Mihm *et al.*, 1981) the same day they were collected.

One-way ANOVA is used to analyze these data; variances among treatments are homogeneous. The row and column effects for the Latin squares are non-significant ($0.25 < P < 0.50$ and $0.10 < P < 0.25$, respectively).

RESULTS

The planulae show no difference ($F_{1,90} = 0.15$; $0.50 < P < 0.75$) in their average number of encounters with either plastic (18.0 ± 16.2) or glass coverslips (16.1 ± 15.5) during the control observations. This ratio of contact between plastic and glass (1.1) does not change among the four time intervals during the eight hour observation period (0.8, 1.6, 1.1, 1.3). The probability of encountering these two substrates when they are simultaneously available to planulae is the same, and the differences in attachment on them in the experiments, below, cannot be attributed to different relative availability of these surfaces to planulae.

The response of planulae when encountering glass or plastic is shown in Figure 1. First, planulae on both materials show similar behavior with respect to coverslip surface: they appear to glide on the upper surface (just as they do on the bottom of their container) slowly rotating about their long axis in a clockwise direction when viewed from their aboral (anterior) pole; but on the under surface they become vertically oriented at a particular spot with their aboral end against it where they continue to rotate clockwise. Change of location is preceeded by a brief quickening of rotation and a rapid lateral movement of 1–2 mm while still vertically oriented, followed by a loss of contact and swimming in an arc-shaped trajectory as shown in Figure 1, prior to resuming a position normal to the surface upon renewal of contact. Second, differences in the duration of rotation (seconds; 0.95 confidence limits) by the vertically oriented planula while against the under surface on glass (8.4; 6.6, 10.6) and plastic (14.0; 10.4, 18.9) is significant ($F_{1,99} = 7.35$; $0.005 < P < 0.01$). This difference is reflected in the number of planulae attaching to these two materials: after twenty-four hours, 30 individuals (7.9%) attach to glass, while 351 (92.1%) attach to plastic. On the under surface, planulae assume an orientation perpendicular to the substrate, and remain in this position longer on plastic than on glass; this results in a greater proportion of planulae attaching to plastic.

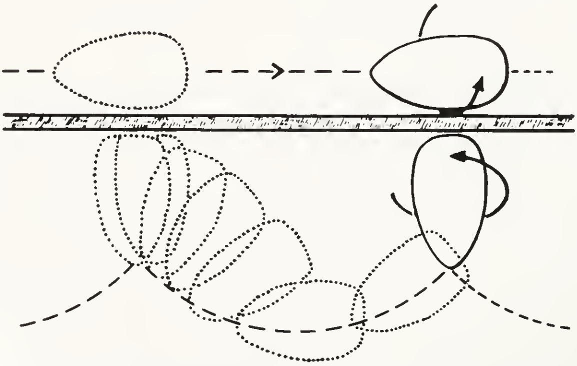

FIGURE 1. The behavior of planulae of *Cyanea* in contact with upper (top of figure) and under (bottom of figure) surfaces. The blunt end (aboral pole) is anteriormost during locomotion. Planulae on the upper surface glide with their long axis parallel to it (dashed arrow), rotating in the direction shown by the solid arrow. The direction of rotation is the same for planulae against the under surface, but they orient perpendicular to it for variable periods of time depending upon the quality of the surface; between these stationary periods of rotation, they move as shown by the dotted outlines along a path described by the curved dashed line.

Aboral contact normal to a substrate is not the sole stimulus leading to attachment. Planulae gliding along a container bottom, or swimming horizontally, collide with the surface of vertically oriented plastic coverslips with their aboral end, but few (348) attach there relative to horizontally held coverslips (5551) ($F_{1,12} = 41.22$; $P < 0.001$). The planulae which attach to the vertical coverslips are equally distributed on either side (165:183), but nearly ten times as many planulae attach to the under surface of horizontal coverslips (5021:530). Though more planulae attach to roughened (920) than to smooth (468) coverslips, there is no difference between them: for vertical coverslips ($F_{1,12} = 0.08$; $P > 0.75$), and for horizontal coverslips ($F_{1,12} = 1.64$; $0.10 < P < 0.25$). Planulae attach to a suitable substrate more frequently if that surface is properly oriented, regardless of its texture (see Discussion).

The temporal sequence of planular attachment to coverslips of plastic or glass is shown in Figure 2. Planulae attach to plastic (at 11.2 hours) 8.4 hours sooner than they attach to glass (at 19.6 hours). Attachment to glass is more variable than that for plastic (C.V. = 66.1% and 20.9%, respectively) and is not normally distributed; the solid curve for attachment to glass (fit by eye) is made parallel to the curve for attachment to plastic. However, even without statistical comparison the difference between attachment to glass and to plastic is clearcut. The actual exponential curve for attachment to glass coverslips (dashed curve, Fig. 2) occurs during the attachment of planulae to the hyponeuston beginning at seventeen hours, to the dish bottom at nineteen hours, and to the upper surface of coverslips at twenty-one hours. Planulae attach to plastic surfaces before they do to those of glass, but during their settlement on glass coverslips, they attach to other surfaces in their experimental container.

Three other comparisons between planular response to plastic and to glass can be made from this experiment of twenty-four hours duration. First, as above, the

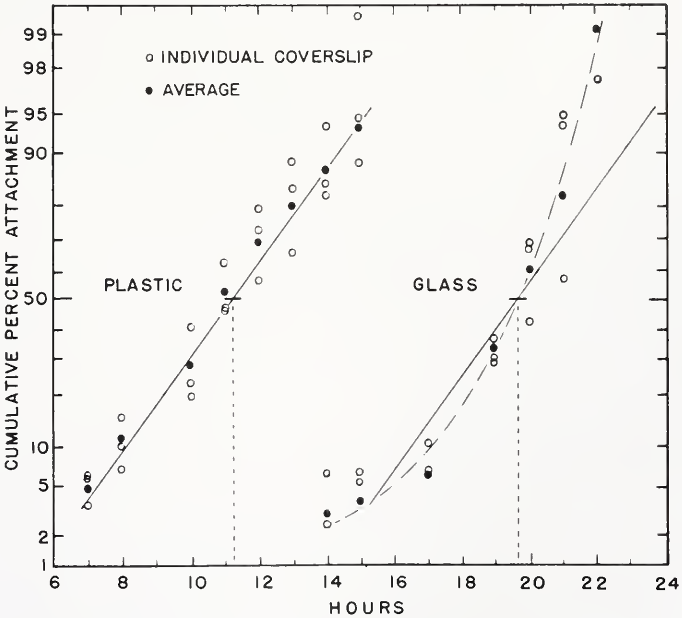

FIGURE 2. The temporal pattern of attachment of planulae of *Cyanea* to plastic and to glass coverslips. Where less than three individual coverslips are shown for an observation period, it (they) equal either 0% or 100%.

number of planulae attaching to plastic (2395) exceeds that for glass (84). Second, only 65 (2.7%) planulae attach to the upper surface of plastic coverslips (also, as above), but the attached planulae are evenly distributed between under and upper surfaces on glass coverslips (40 and 44, respectively). Third, equal numbers of planulae attach to the dish bottom beneath the plastic and glass coverslips (139 and 134, respectively) showing again (see above), but indirectly, that both coverslip types are equally accessible to the planulae.

The contact angle, or surface wettability, affects planular attachment (Fig. 3). Planulae vary in their attachment to surfaces which exhibit a contact angle less than 19° (lens cleaner-coated coverslips, 17.5 ± 5.1°; opal, 17.3 ± 1.9°; and mica, 18.9 ± 1.8°), and at angles greater than this, but including the data for opal for the lowest point, their relative attachment to the substrates available to them directly parallels the size of the angle ($\hat{Y} = 52.4 + 2.7X$; $0.005 < P < 0.01$). The diameter of the planulocysts formed by the attaching planulae is also correlated with contact angle: the cysts formed on glass (230.3 μm) are smaller ($F_{1,196} = 6.62$; $P \simeq 0.01$) than are those formed on plastic or either of the siliclad-treated coverslips (300.0 μm; 299.0 μm; 295.9 μm, respectively); there is no difference in size among these latter planulocysts ($F_{2,196} = 0.68$; $0.25 < P < 0.50$). The contact angle for roughened plastic coverslips (80.1 ± 2.2°) on which more planulae attach (see above) is larger than that of their smooth counterparts (67.6 ± 3.5°) and is close to the contact angle determined for siliclad-coated coverslips (81.6 ± 3.6°) on which most planulae settle (Fig. 3). On natural substrates to which planulae are exposed in the field, the contact angle on fresh scallop shell (18.8 ± 3.0°) approximates that for opal, mica, and lens cleaner-coated coverslips upon which settlement is relatively low and variable; wave-worn

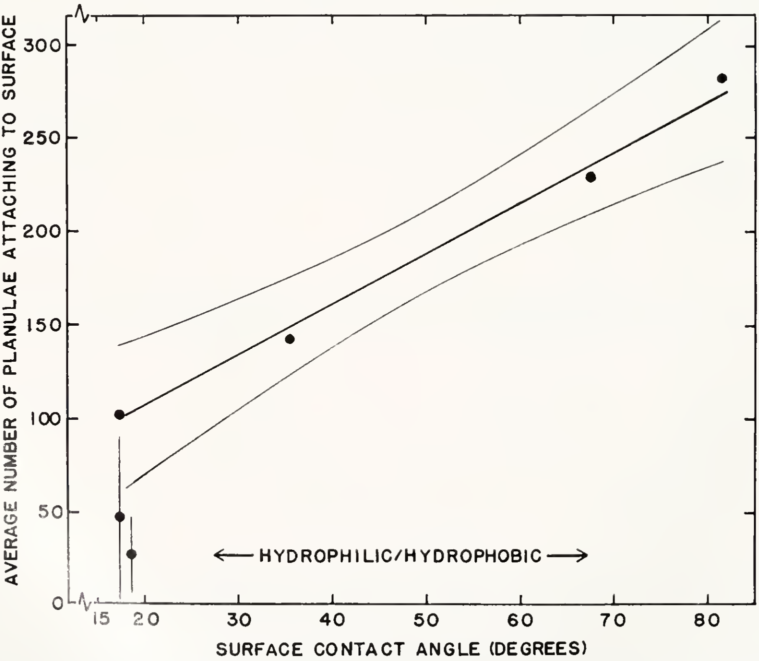

FIGURE 3. The average attachment (and 0.95 confidence limits) of planulae of *Cyanea* exposed sixty hours to surfaces with different contact angles. See text for identification of surface materials.

shells (30.9 ± 7.5°) and non-eroded shells (35.9 ± 5.1°) show larger contact angles similar to that for glass (35.9 ± 3.7°); and the largest contact angle for organic substrate is found on eroded scallop shells bearing planulocysts (52.4 ± 2.6°). *Cyanea* planulae attach most readily to inorganic surfaces which are relatively hydrophobic, and this is apparently also true for organic substrates which they normally encounter in the field (See Discussion).

DISCUSSION

Cyanea planulae are influenced by the relative hydrophobicity of hard surfaces, not only in their actual attachment but also in the correlates of attachment (duration of inspection, time required to attach, size of encysted planulae). The more non-wettable or hydrophobic the surface, indicated by large contact angles, (1) the longer the period of inspection, (2) the shorter the time to attach, (3) the higher the frequency of attachment, and (4) the larger the encysted planulae. The interpretation of the relationships among the process of inspection, the time to attach, and actual attachment is straightforward; the size of the cyst, on the other hand, is a manifestation of surface properties associated with adherence once contact is achieved. The cyst produced by planulae upon attachment is a measure of the size attained when their attachment is complete, and may be considered to be a multicellular analogue to metazoan cells in tissue culture. Such cells exhibit greater spreading on surfaces to which they adhere (*e.g.*, Weiss, 1961; Weiss and Blumenson, 1967; Baier, 1980), and planulocysts are larger in diameter on surfaces to which adherence is most readily obtained (hydrophobic surfaces). Planular response to clean, smooth surfaces in the laboratory demonstrate physical constraints imposed upon their attachment to them; this has been shown for other marine larvae.

The response of planulae to surfaces exhibiting different wettability is not unique. The results obtained by Eiben (1976) and Mihm *et al.* (1981), both of whom used larvae of Bryozoa, show this phenomenon. Eiben (1976; see also Müller *et al.*, 1976) describes a predictive model to account for his observations using the larvae of *Bowerbankia* exposed to surfaces of different wettability. This model is based on a well known principle of thermodynamics, that in any system surface free energy will tend to a minimum (*e.g.*, Steinberg, 1964). Larvae cannot settle upon surfaces with high wettability (small contact angles), for this combination of larval surface and substrate does not lead to the greatest reduction in surface free energy; the larvae cannot overcome the bonding strength of the particular configuration of water molecules in contact with an hydrophilic surface (*e.g.*, Tanford, 1973). The largest contact angle at which larvae cannot attach Eiben (1976) calls the "critical angle;" the critical angle for *Bowerbankia* is 17°, and for *Bugula* (Mihm *et al.*, 1981) it is at least as large as 45°. No unambiguous critical angle is demonstrated for planulae over the range of contact angles examined, but the variation in their response to contact angles between 17° and 19° if not due to other causes might indicate that they could not attach to surfaces exhibiting a slightly greater wettability. While the details differ, the planulae of *Cyanea* show a limited capacity for attachment to highly wettable surfaces in general agreement with the model proposed by Eiben (1976).

Another prediction of Eiben's (1976) model is that larval attachment will be uniformly high on surfaces with contact angles exceeding the critical angle. He shows this for *Bowerbankia,* and the data for *Bugula* (Mihm *et al.*, 1981) also support such an "all-or-nothing" response. However, over the range of contact angles permitting attachment, the planulae of *Cyanea* show a response proportional to the hydrophobicity of the surface. This pattern for planulae is probably due to differences in experimental procedure rather than reflecting disagreement with the model of Eiben (1976) and the results of Mihm *et al.* (1981) for Bryozoa. Their data represents the proportion of larvae attaching out of the total number of larvae exposed for short duration, to a single substrate; mine, the relative numbers of planulae selecting a particular substrate when presented with a choice among several during prolonged exposure. The different time element is dictated by larval characteristics: the short-lived, synchronously settling bryozoan larva compared to the longer-lived, asynchronously settling planulae. However, the correlation in the numbers of planulae of *Cyanea* attaching to surfaces of different wettability emphasizes the relationship between their attachment and this surface property of solids.

The attachment of the larvae of *Bowerbankia* to non-wettable surfaces is described by Eiben (1976) as an exergonic process. This does not completely explain the interaction of planulae with hydrophobic surfaces, for it leads to the expectation that both their behavior and frequency of attachment to upper and to under surfaces of plastic coverslips, or to vertically held plastic coverslips, should be more alike than is observed. The temporal sequence of planular attachment shows planulae finally settling (literally?), and in an atypical location, on the upper surface of coverslips (and on the dish bottom) only when their metabolic reserves become largely depleted (?) relatively late during their free-swimming phase. Planulae do not indescriminately respond passively to suitable substrate, but only to this substrate when it is horizontally oriented and they approach it from below.

Substrates in the field exhibit both different textures and variable degrees of fouling; both roughness and fouling alters the wettability of the original surface. Though the accurate measurement of contact angle on rough surfaces is difficult (Baier *et al.*, 1968), empirical results generally agree with theory: roughening the surface of a solid lowers its surface energy (Johnson and Dettre, 1964). The change in wettability that altering the texture of coverslips effects, and the attachment of planulae of *Cyanea* and of other Scyphozoa to them, is shown in Table I. Planulae of *Cyanea, Aurelia,* and *Chrysaora* attach to a greater extent on the rough, more hydrophobic surface. While only Brewer's (1976a) data shows significantly more planulae attaching to rough surfaces, these consistent results suggest that planular attachment to roughened substrate might represent their response to the change in wettability caused by surface alteration rather than to texture, *per se.* This may be why scyphozoan larvae are not found more frequently on rough substrate in the field (Cargo, 1979; Brewer, in prep.) for even a smooth surface with a large contact angle, for whatever cause, would provide an equally suitable surface upon which to settle.

The smooth inner surface of bivalve shells is a common site for the attachment of planulae of *Cyanea* in the field (Brewer, in prep.), but clean shells exhibit small contact angles equivalent to those of inorganic surfaces relatively unfavorable for planular settlement; this may explain why repeated attempts to obtain planular attachment on such shells in the laboratory have met with only sporadic success (Brewer, unpubl.). Mihm *et al.* (1981) show this for clean oyster shell finding it a poor substrate for the attachment of *Bugula.* However, clean surfaces quickly become fouled with an organic film followed by bacterial colonization when immersed in sea water (Zobell, 1943; Baier, 1970, 1980; Fletcher, 1979). The composition of adherends may differ qualitatively and/or quantitatively depending upon the wettability of the initial surface (Baier, 1980), with the nature of the organic film influencing bacterial attachment, and the two together, that of larvae: oyster shell allowed to become filmed and colonized with bacteria exhibit low wettability and a correspondingly high attachment to it by *Bugula* (Mihm *et al.*, 1981). The differences in the wettability of scallop shells becoming increasingly more hydrophobic with length of immersion is indirect evidence

Table 1

The relative number of planulae of Scyphozoa attaching to rough and to smooth coverslips

Species (reference)	Number of planulae on coverslips (contact angle) Rough (80.1°)	Smooth (67.6°)	Ratio Rough/smooth
Cyanea (this study)	920	468	1.97
Cyanea (Brewer, 1976a)	18603	11537	1.61
Cyanea (Brewer, unpubl.)	852	469	1.82
Aurelia (Brewer, 1978)	237	191	1.24
Chrysaora (Cargo, 1979)	98136	71010	1.38

that surface changes (through erosion) and/or the presence of films, altering their wettability, is necessary for the attachment of the planula of *Cyanea* in the field. Planulae have never been found attached to fresh shells, and the attached stages of *Cyanea* are most commonly encountered on older, eroded ones showing a bronzy iridescence on their inner surface (pers. obs.).

The correlation between larval settlement and surface wettability describes permissable conditions for their attachment, but not the mechanism of adhesion nor the stimulus for subsequent metamorphosis. The close contact of small and weakly powered larvae allowed by nonwettable substrates permits the occurrence of an effective physical stimulus, shearing forces exerted on cilia, initiating the final attachment of *Bowerbankia* (Eiben, 1976) and of *Hydractinia* (Müller *et al.,* 1976). Perhaps such proximity brought about by appropriately wettable surfaces is a requisite for other larvae for which chemical mechanisms are implicated: for example, the enzyme-like reaction between surface lectins of larvae and the polysaccharides produced by bacterial films proposed by Kirchman *et al.* (1982b) for the larval attachment of Bryozoa. The mechanisms, physical, chemical, or whether a combination of both, initiating the transformation of the planulae of *Cyanea* into a lenticular-shaped disc when completely attached (see Brewer, 1976a; Fig. 1) are not known.

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THE EFFECT OF GROUP INTERACTIONS ON THE DEVELOPMENT OF SIZE DISTRIBUTION IN *MACROBRACHIUM ROSENBERGII* (DE MAN) JUVENILE POPULATIONS

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ABSTRACT

The distribution of body size among juvenile prawns, *Macrobrachium rosenbergii* (de Man), was assessed by comparing communally reared populations with singly raised individuals. Prawns reared in a group showed a relative excess of size variation, primarily associated with the appearance of two size classes which failed to appear among juveniles raised in isolation: 1) exceptionally fast-growing individuals, termed "jumpers," and 2) "laggards," which are generated in the presence of jumpers and represent a group of severely growth-repressed individuals. These results suggest that the variance of attained sizes of juveniles in a *M. rosenbergii* population is more affected by interactions within the prawn group than it is by genetic differences in growth potential of the individual prawns.

INTRODUCTION

Heterogeneous growth in aquatic species has been associated with factors that are intrinsic (Newkirk *et al.,* 1977), environmental (Wilbur and Collins, 1973), or social (Brown, 1946; Symons, 1972). In the present study we examine the growth of juveniles of the freshwater prawn *Macrobrachium rosenbergii* (de Man) and assess the extent to which an individual growth rate may be enhanced or suppressed by the presence of other individuals of the same species. Although growth suppression through competition has been documented (Wilbur and Collins, 1973; Wohlfarth, 1977), stimulation of growth which is observed under communal conditions but not in isolation is still a puzzling biological phenomenon.

We have chosen the freshwater prawn *M. rosenbergii* as the model organism for this study since, although the average weight of newly metamorphosed post larvae is 0.009 g with a small variance ($\sigma^2 = 6.0 \times 10^{-6}$ g, Sandifer and Smith, 1975; Malecha, 1977), the distribution changes gradually with time, as both variance and skewness increase. A "leading tail" in the distribution curve, containing individuals which are larger than the bulk of the population, is formed. Fast growing individuals, henceforth termed "jumpers," can grow as much as fifteen times larger than the population mode within a period of 60 days from metamorphosis (Willis and Berrigan, 1977; Ra'anan, 1982).

In a previous study on the same organism (Ra'anan, 1982; Ra'anan and Cohen, 1983) we showed that when populations of newly metamorphosed post larvae are reared at various stocking densities the average growth rate, the degree of size variation, and most important, the degree of skewness, all decreased with increasing density.

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Our results contrasted with those obtained in similar studies on other aquatic organisms (Wilbur and Collins, 1973; Wohlfarth, 1977) where positive asymmetry of the weight distribution curves was more pronounced under the restricting growth conditions of high initial density, but diminished under improved conditions as initial stocking density decreased. Their results were interpreted as evidence for the effects of competition. On the other hand, our observations (Ra'anan and Cohen, 1983) led us to conclude that the appearance of jumpers in *M. rosenbergii* is not primarily a result of competition, if at all, but of factors intrinsically associated with population development. Sandifer and Smith (1975) suggested that this growth pattern may be caused primarily by genetic rather than environmental factors. However, Malecha (1977) reasoned that the size variance is due more to social interactions than to the segregation and independent assortment of genes controlling growth.

In order to differentiate between the relative contributions of genetic and social factors to the observed heterogeneous size distributions, we compared a communally reared post larval population with a parallel set of individually raised prawns. We used the change with time in the variance of the ln-transformed weights as a measure of intrinsic heterogeneity in the growth rates of the animals in the alternative growth regimes. If intrinsic factors are directly responsible for the variation in growth rates, we would expect a similar increase in the logarithmic variance in both the individual and the communal rearing systems. However, if interactions within groups are responsible for the observed growth heterogeneity, regardless of mechanism, the logarithmic variation of weights of individually reared juveniles should tend to remain constant with time while that of the communally grown animals should increase.

In this paper we establish the existence of growth enhancement in group-reared juveniles, and show that the observed rapid growth of the jumpers does not occur when prawns are raised individually. The wide size variance cannot therefore be attributed to genetic differences in the individuals' growth potential alone. We conclude that the growth stimulation is associated with the communal environment which is specifically responsible for the observed size heterogeneity and the appearance of jumpers in *M. rosenbergii* juvenile populations.

MATERIALS AND METHODS

Newly metamorphosed post larvae (PL) were obtained from the experimental hatchery at the Hebrew University of Jerusalem, operated by Aquaculture Production Technology (Israel) Ltd. PL from a single brood were divided into two experimental groups. One group was stocked individually in separate 4.5 liter aquaria (15 cm × 15 cm × 20 cm) with built in biofilters and no water connection between them (n = 120), while the other group (n = 180) was stocked into a tank of 720 liters (100 cm × 100 cm × 80 cm) with an external biofilter, for communal growth. The small aquaria were kept in the same room under equivalent controlled temperature (26 ± 1°C) and light regime (12L:12D) as the large communal tank. Both groups of prawns were fed daily with an excess of live *Daphnia* and ground fresh fish. Residual food and other particulate matter were removed weekly by siphoning. Additional submerged substrates, in the form of plastic boards, were placed inside the communal growth tank so that stocking densities per volume and per surface area were equivalent in both systems (1 PL per 4 liters and 1 PL per 0.08 m^2).

Since the total space available for each animal reared under communal growth conditions (720 liters) was much larger than that available for each individually reared PL (4 liters), a control experiment was designed in order to examine whether this factor alone could affect the growth rate of the isolated juveniles. In this experiment both communal and isolated prawns were monitored in identical aquaria (9 liters, 15 cm × 30 cm × 20 cm). Total available space was now identical for each of the animals, although their stocking density was greater under communal conditions (1 PL per 0.9 liters and 0.013 m^2) than it was for the isolated prawns (1 PL per 9 liters and 0.13 m^2).

The changes with time in the size distribution curves of both individually and communally grown populations in the two experiments were followed by weighing all animals individually once a week during 63 days. PL were weighed in water, using an automatic tare Sartorius balance. Data were analyzed on two levels. First we estimated the changes occurring in the variance and the skewness of the ln-transformed

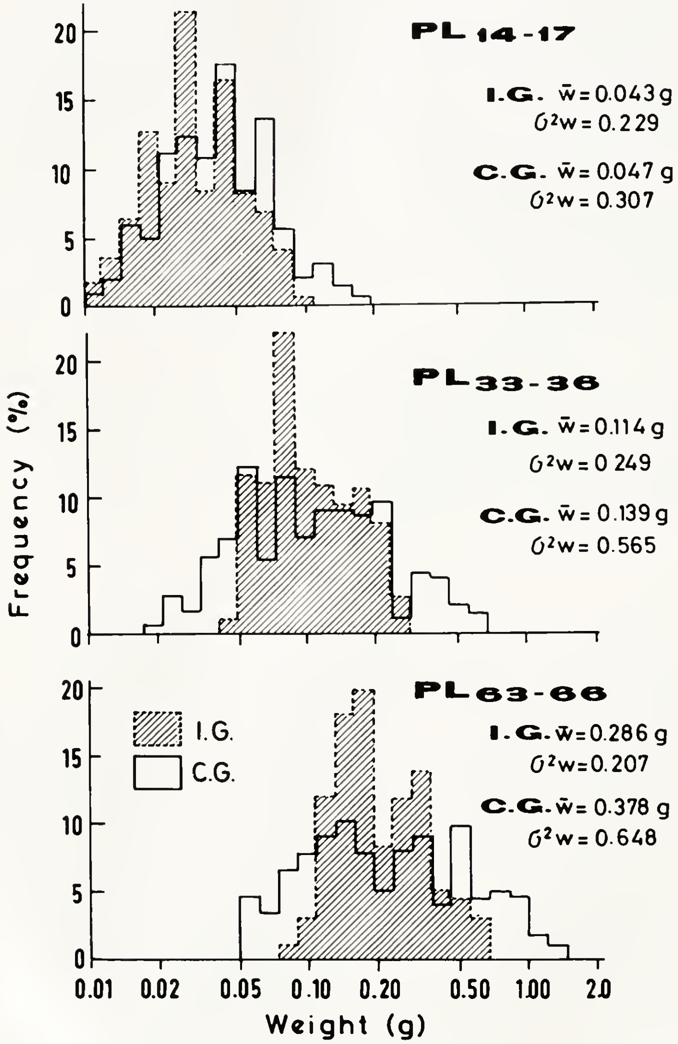


FIGURE 1. Frequency histograms of body weights of post larvae reared under individual (I.G., n = 120) and communal growth (C.G., n = 180) conditions. Upper, 14–17 days post metamorphosis; middle, 33–36 days; bottom, 63–66 days post metamorphosis.

weights in order to assess the degree of variation in growth within the respective populations. These parameters were calculated by the SPSS frequency routine for each weekly weighing. Next we calculated the individual relative growth rates, R, ($R = \frac{1}{T - t} \ln \frac{W_T}{W_t}$, where W_T and W_t are body weights at times T and t, $T > t$, according to Ricker, 1958; Wilbur and Collins, 1973) for all the juveniles in both populations. Comparative estimates of the R value distributions of communally and individually reared juveniles permit a direct evaluation of the respective growth heterogeneities.

RESULTS

The development of size variation

The frequency histograms of body weights for the individually reared post larvae (Fig. 1) and their statistical analyses (Table I) approximated a lognormal distribution. While the average weight increased with time, the logarithmic variance remained relatively constant throughout the experiment. Under communal growth conditions, however, both the average weight and the logarithmic variance continued to increase with time, and the initial lognormal distribution became positively skewed. The degree of skewness increased until the fifth week and then decreased slightly. The rate of increase of the average weight in both groups was similar until the sixth week (Fig. 2). Afterwards, the increase in average weight in the communally reared population exceeded that of the singles group. A comparison of the uppermost decile of the population by weight, the lowest decile, and the median value of the distribution in both populations (Table II) shows that the fastest growth is attained by the largest communally reared juveniles, to an average weight of 1.19 g, whereas the largest isolated individuals grew to an average of only 0.57 g during the 63 days. Growth of the smallest communally grown animals began to lag about four weeks after metamorphosis, while the distribution median value in both populations advanced at similar rates.

In the equal space per PL control experiment, size distributions for communal and individual PL were compared two and five weeks after metamorphosis (Fig. 3).

TABLE I

Changes with time in the mean ($\bar{X}$), standard deviation (S.D.), and skewness (Sk.) of weights in experimental juvenile prawns

Days after metamorphosis	Individual growth (n = 120)			Communal growth (n = 180)		
	$\bar{X}$ (g)	S.D. (σlnW)	Sk.	$\bar{X}$ (g)	S.D. (σlnW)	Sk.
14	0.043	0.478	−0.024	0.047	0.554	0.067
21	0.077	0.470	−0.032	0.077	0.647	0.107
28	0.096	0.464	0.079	0.094	0.682	0.178
35*	0.114	0.499	0.047	0.139	0.751	0.196
42*	0.202	0.482	0.120	0.241	0.770	0.242
49*	0.221	0.470	0.099	0.287	0.780	0.221
56*	0.250	0.471	0.045	0.327	0.788	0.184
63*	0.286	0.455	0.052	0.378	0.805	0.154

* Differences between means of individually and communally reared populations are statistically significant (*t*-test, $\alpha = 0.05$).

TABLE II

Increase in average weights (g) of the largest (upper 10%), smallest (lowest 10%), and the median body weight experimental subjects reared under individual (I.G.) versus *communal (C.G.) growth conditions*

	10% Largest		Median		10% Smallest	
Days after metamorphosis	I.G. (n = 10–12)	C.G. (n = 16–18)	I.G.	C.G.	I.G. (n = 10–12)	C.G. (n = 16–18)
14	0.08*	0.15	0.031	0.041	0.017	0.018
21	0.16*	0.25	0.066	0.063	0.031	0.027
28	0.20*	0.39	0.080	0.073	0.042	0.031
35	0.28*	0.47	0.109	0.103	0.061*	0.037
42	0.38*	0.72	0.152	0.160	0.083*	0.062
49	0.48*	0.87	0.177	0.191	0.097*	0.073
56	0.55*	0.99	0.208	0.223	0.114*	0.081
63	0.57*	1.19	0.227	0.252	0.138*	0.084

* Differences between means are statistically significant (*t*-test, $\alpha = 0.05$).

The percent coefficient of variation ($\text{P.C.V.} = 100 \times \frac{\text{S.D.}}{\text{W}}$) of communal PL reached 44.2, while that of the individual PL reached 22.0. Note that the difference in P.C.V. occurred during the first two weeks. Most of the enhancement of growth shown by the largest individuals in the communal group occurred in both experiments within the first two weeks and the corresponding lag in growth rate demonstrated by the smallest individuals communally reared became apparent only in later stages.

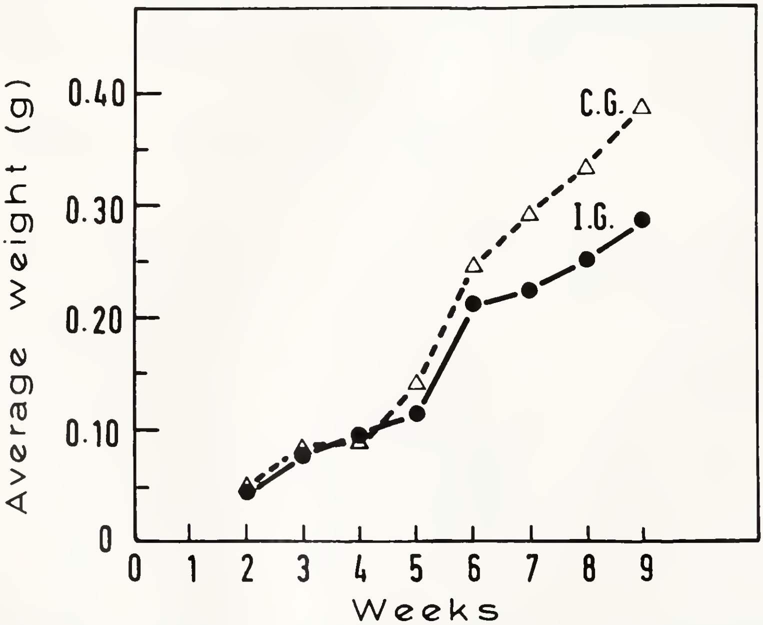
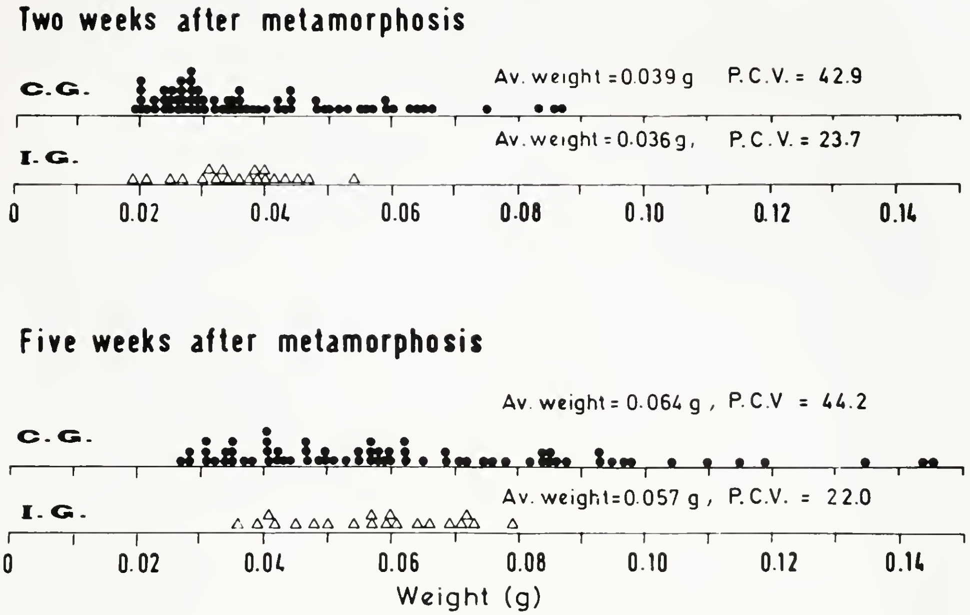


FIGURE 2. Rate of increase in average weight with time of individually (I.G.) and communally (C.G.) reared post larvae. Differences between means are statistically significant (*t*-test, $\alpha = 0.05$).


Two weeks after metamorphosis

Five weeks after metamorphosis

FIGURE 3. Weight distribution of juveniles reared communally (C.G., n = 70) and individually (I.G., n = 20), in the control experiment (equal space for both cultures), two and five weeks after metamorphosis. $\text{P.C.V.} = 100 \times \dfrac{\text{S.D.}}{\text{average weight}}$

Clearly, heterogeneity of growth in juvenile populations, as evaluated by the increased variance of the ln-transformed weight distributions with time, is much higher for communally reared juveniles than for those reared as individuals. Moreover, the largest individuals among the communal juveniles grew faster than the largest ones that were individually grown. This was also observed in the equal space control population, clearly indicating that the size of the container was not a limiting factor in the growth of the individually reared PL.

The distribution of relative growth rates

We calculated relative growth rates of individuals in both the isolated and the communal rearing systems in order to directly measure the variation of R values in the respective conditions, as well as to learn about the changes of R values with time in isolated *versus* communally grown prawns. In the communal culture we noticed that certain marked individuals maintained their relative size ranking, and on this basis we inferred that such stability is fairly general. Variance of 1.12 and 0.72 of communally and individually reared populations, respectively, two weeks after metamorphosis, decreased to 0.34 and 0.23, respectively, by the seventh week (Fig. 4). The R distribution of the communal population was wider, and was positively skewed at the initial growth period as compared with a narrower, more normal distribution attained by the individually reared juveniles. R values of the deciles by weight in the population, were plotted against the respective initial body weights of juvenile prawns reared communally between 14 to 21 and 56 to 63 days after metamorphosis (Fig.

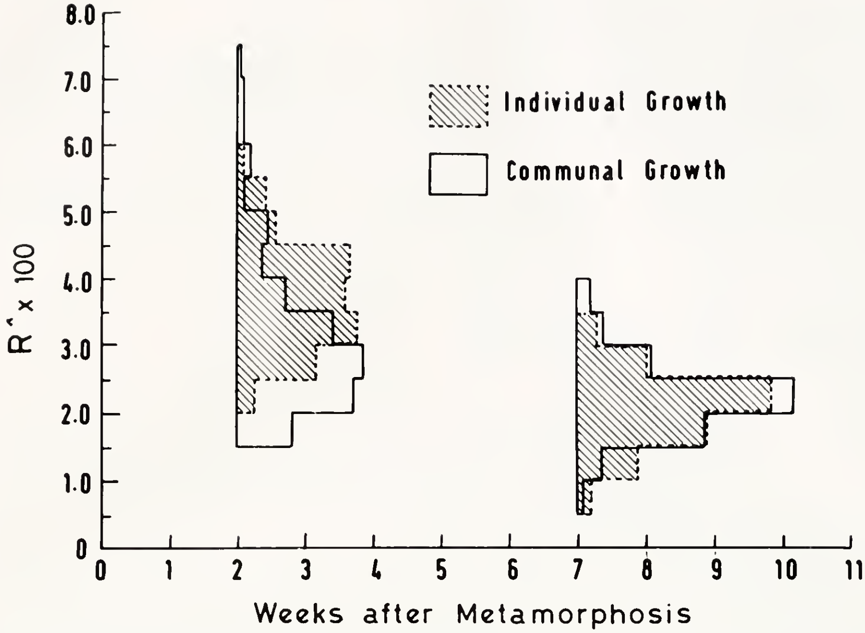


FIGURE 4. Frequency distributions of relative growth rates (R) in individually and communally reared juveniles, two and seven weeks after metamorphosis.

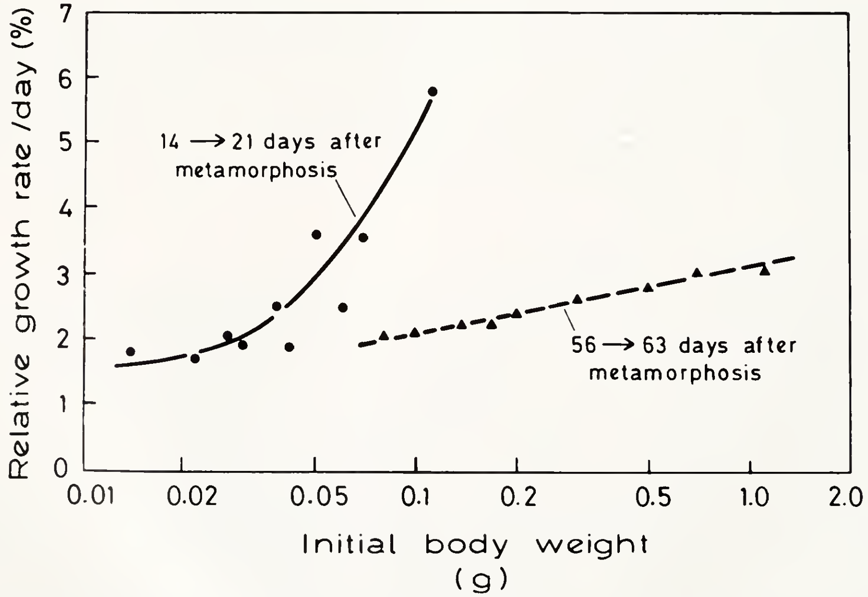


FIGURE 5. The relation between relative growth rates of body weight and initial body weight of communally reared juvenile prawns.

5). We find that R was positively correlated with the initial body weight in both periods, and that this correlation was more pronounced during the first time interval.

Discussion

The development of size variation

Growth in juvenile populations, as measured by the increase in variance of the ln-transformed weight distributions with time, is much higher for communally reared juveniles than for those reared as individuals. A similar phenomenon was observed by Malecha (pers. comm.) when larger juveniles, averaging 1.0 g, were selected for continual growth in separated cells.

Communally raised carps also had larger size variance than those reared individually (Nakamura and Kasahara, 1957; Yamagishi, 1962). The isolated carp, however, grew at rates similar to those exhibited by the fastest growing individuals under communal growth conditions. This result was explained by the absence of competition which had apparently inhibited growth of the smaller group-reared fish. In contrast, the excess in average weight of the communally reared *M. rosenbergii* juveniles relative to that of the individually grown animals (Fig. 2) indicates that jumpers' superior growth rates result from the presence of some stimulus which is lacking under conditions of individual isolation. This is supported by the observation that the decile containing the largest individuals in the communal group reached an average weight much higher than that achieved by the comparable decile of the isolated individuals during the same interval (Table II). Lee and Fielder (1983) reported an identical phenomenon in the prawn *Macrobrachium australiense,* ("Under laboratory conditions, group held juveniles produced more biomass than equivalent numbers of individually held prawns kept for the same time. However, the individually held prawns had a more uniform growth as shown by smaller standard errors in their mean sizes. . . . ") without referring to the growth stimulation which is the basis of the distribution pattern they observed. Allee (1951) illustrated many examples of the benefits of being in a group to the well being of the individual. He discussed cases of social facilitation leading to improved survival probabilities, improved chances of finding a mate, group stimulation of food consumption, and even improved learning capabilities. He also defined social behavior as any type of response which differs from that which would be shown if the animals were solitary. The greater the difference between their behavior when grouped than when isolated, the more social they are. Growth stimulation was also described by Uematsu (1971a, b) in the guppy, *Poecilia reticulata* (Peters), where the social facilitation of growth observed in the communal rearing system was associated with changes in feeding behavior and rates of respiration. In the himedaka (*Oryzias latipes*) social facilitation of growth was recognized and termed "the presence recognition effect" (Uematsu and Takamori, 1976).

Sastry and French (1977) and Aiken and Waddy (1977) found a strong correlation between the size of the container and the growth rate of the housed lobster, *Homarus americanus.* Van Olst and Calberg (1979) estimated that in order to avoid reduction in growth rate, the area of the container must be approximately the square of three times the body length of the lobster, or $(3BL)^2$. The maximum body length we observed in our experiment was 2.5 cm, which, in terms of the above formula, would dictate a surface area requirement of no more than 56 cm^2, whereas, in fact, the bottom area available to each animal was 300 cm^2. Nevertheless, in order to ascertain that the growth of the largest individuals in the separated aquaria was not inhibited by the size of the container or some other variable associated with it, the second experiment was conducted, in which both individuals and groups were raised in identical aquaria.

The results here were consistent with the main experiment. Communally reared jumpers grew significantly faster than the fastest individually housed juveniles, even though the space per animal available to the jumpers was much less than the space available to the singles. The possibility that container size adversely affected the growth of the largest individually reared prawns is therefore ruled out.

The influence of communal growth on individual size development in prawns is effective at both ends of the size distribution. The advent of jumpers is a consequence of growth facilitation and, thereafter, the lowest decile of the population specifically undergoes a growth lag (Fig. 1, Table II). The later appearance of the slow growth in small individuals is also correlated with the decrease in skewness of the size distribution curve starting from the fifth week (Table I). These observations suggest that such retarded growth might be due to a cumulative suppressive effect borne by small individuals in the presence of jumpers. We have shown elsewhere (Ra'anan, 1982) that after each of several periodic removal of jumpers from a communal growth system the remaining population immediately underwent a period of enhanced growth until a new group of jumpers developed.

In summary, the appearance of jumpers and the suppression of laggards as expressed in group culture, and their absence in isolation, support the notion that social factors (as defined by Allee, 1951) control size variation in *M. rosenbergii* juvenile populations more effectively than genetic factors governing potential individual size.

The distribution of relative growth rates

R ordinarily decreases with age and size (Brown, 1946; Yamagishi, 1962) and, indeed, the growth rates of the individuals in this experiment decreased with time as the animals' weights increased, under both culture conditions applied. The variance in R values at the initial growth period was more pronounced for the communally than for the individually reared populations, but with time it, too, decreased.

We also found a positive correlation between the R values and the positions of the individuals with respect to rank in size within the population, a phenomenon which may be fairly general. Brown (1946) had reported that individual relative growth rates in trout fry were usually higher for larger juveniles. Thus, the order of descending weight was also an order of decreasing relative growth rate for the group. This phenomenon was analyzed mathematically by Koyama and Kira (1956), who proposed four types of mathematically derived size frequency distributions, assuming exponential growth by individual plants in a population. They referred to the condition in which initial weight (W_0) and R have normal distributions, and in which R is positively correlated with size, as the N-N correspondent type. Just such a positive correlation was obtained between initial body weight and relative growth rates of our juveniles throughout the eight week period after metamorphosis (Fig. 5). The marked increase in the positive asymmetry of the juvenile weight distribution under communal growth conditions can be attributed to the enhanced growth rates of the larger juveniles, and especially the largest among them.

Since the correlated size ranking and relative growth rates in *M. rosenbergii* juveniles are subject to interactions within populations, the question arises as to what extent these parameters may be fixed or reversible traits in the ontogeny of individual juveniles. Experiments on this problem are in progress.

ACKNOWLEDGMENTS

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LIFE HISTORY STUDIES ON THE RED SEA SOFT CORAL *XENIA MACROSPICULATA* GOHAR, 1940. I. ANNUAL DYNAMICS OF GONADAL DEVELOPMENT

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ABSTRACT

Xenia macrospiculata Gohar, 1940 is a dominant xeniid species on the coral reefs of the Gulf of Eilat. The colonies are dioecious and the gonads develop along the four lateral and two sulcal mesenteries, except for the anthocodial part of the polyps. The population starts to reproduce at a markedly early age of two years and the percentage of sexually mature colonies increases with coral size. The sex ratio of the population is significantly skewed toward a higher abundance of males.

A synchronization of gonadal development occurs among the polyps within a colony. In every annual cycle the development of initial gonadal primordia starts in September or October and it lasts until April–May. In addition to these gonads, more oocytes and sperm sacs continue to appear along the mesenteries of male and female colonies during the succeeding 7–8 months. Due to the prolonged period of gonadal initiation, the population contains colonies in various stages of development. The successive maturation of gametes starts every annual cycle in May and lasts for 4–5 months. During this time polyp cavities become filled with an unusual compact arrangement of various sizes of oocytes or sperm sacs. The early age of first reproduction combined with the prolonged gametogenic season increase the reproductive potential of *X. macrospiculata* and contribute to its high abundance at the studied reefs.

INTRODUCTION

The dominance of soft corals of the family Xeniidae at the coral reefs of the northern Red Sea has been noted by Fishelson (1970), Schuhmacher (1974), and Benayahu and Loya (1977, 1981). In this area there are 20 species of *Xenia* (Benayahu and Loya, in prep.) included in a diverse assemblage of soft corals. *Xenia macrospiculata,* one of the most abundant xeniids in the Red Sea, has been described by Gohar (1940a) from the reefs of El-Ghardaqa (Egypt); its present zoogeographical distribution extends to various localities along the Red Sea and Madagascar (Verseveldt, 1971). The aggregations of *X. macrospiculata* create large beds that monopolize the reef substrate (Fig. 1a). In many shallow reef zones their coverage is very high, exceeding 70% of the surface area. In addition, colonies of *X. macrospiculata* exhibit a special mode of active movement, which enables them to utilize vacant space rapidly (Benayahu and Loya, 1981).

Studies on Red Sea xeniids with an emphasis on the reproduction of *Heteroxenia fuscescens* were carried out by Gohar (1940a, b) and Gohar and Roushdy (1961). Yet, no quantitative study was conducted on the annual dynamics of gonadal development of any *Xenia* species, except for the scant information given by Ashworth

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(1899) and Hickson (1931). Similarly, long term and quantitative aspects of the life history of xeniid populations are lacking.

This paper deals with the life history of *X. macrospiculata* in shallow water (3–5 m) and in a deeper reef zone (27–30 m). It is the first quantitative account on the sexual reproduction of a xeniid coral concerned with sex ratio, colony size at reproduction, and annual dynamics of male and female gonadal development.

Materials and Methods

Two reef areas were selected at the Gulf of Eilat for the study. A very dense population of *X. macrospiculata* is found at Muqebla', 12 km south of Eilat (Fig. 1a). At this coral reef, the shallow population (3–5 m) was studied. The deep water population (27–30 m) was sampled at the reef in front of the Marine Biological Laboratory of Eilat (M.B.L.). The two reefs were studied from March 1977 until July 1981. Every month 20 large colonies were collected randomly from each locality by SCUBA diving. The colonies were carefully detached from the substrate and preserved in 4% buffered formalin. In the laboratory the colonies were sectioned longitudinally (Fig. 2a) and examined under a binocular microscope for the presence of gonads, sex determination, and the percent of space along the edges of the mesenteries occupied by oocytes or sperm sacs. We also examined possible synchronization in the reproductive state of mesenteries derived from one polyp, in polyps from the same branch, and in branches of the same colony. Fine-pointed forceps were used to prepare wet mounts of mesenteries from each colony so that the size range of oocytes and sperm sacs could be measured. In order to distinguish between colonies with male or female initial gonadal primordia (IGP), histological sections were examined. Formalin-fixed colonies were rinsed with fresh water and then transferred into 70% methyl alcohol. Decalcification was carried out for 10 min by formic acid and sodium citrate (Rinkevich and Loya, 1979a). Paraffin sections (10 μm) were stained with Delafield hematoxylin and eosin.

Xenia macrospiculata exhibits a diurnal pattern of polyp and colony contraction (Fig. 1b), and hence it is difficult to measure the size of living colonies. In order to determine the relationship between size and the presence of gonads, colonies of various sizes were sampled prior to the breeding season of 1978 and 1979, on both shallow and deep reefs. The colonies were preserved in formalin, which caused their complete contraction. Water displacement from a graduated cylinder was used to determine the volume of each colony: every colony was introduced into a cylinder and the amount of the displaced water was measured by pipette with an accuracy of ±0.1 ml. In addition, these colonies were sectioned and their sexual maturity was recorded.

RESULTS

General features of the colonies and the gonads

X. macrospiculata colonies are small sized soft corals, attaining a height of only a few cm (Fig. 1c). Living corals are creamy brown, although numerous sclerites often cause a chalky appearance, especially within the shallow water population. Each colony is attached to the substrate by a basal part of its stalk (Fig. 1c). Many colonies are composed of a few branches, each terminating with a polyp-bearing capitulum. The polyp cavity runs from its free part above the capitulum (anthocodium) and passes into the syndete, the coenenchymal part of the stalk (Figs. 1c, 2a).

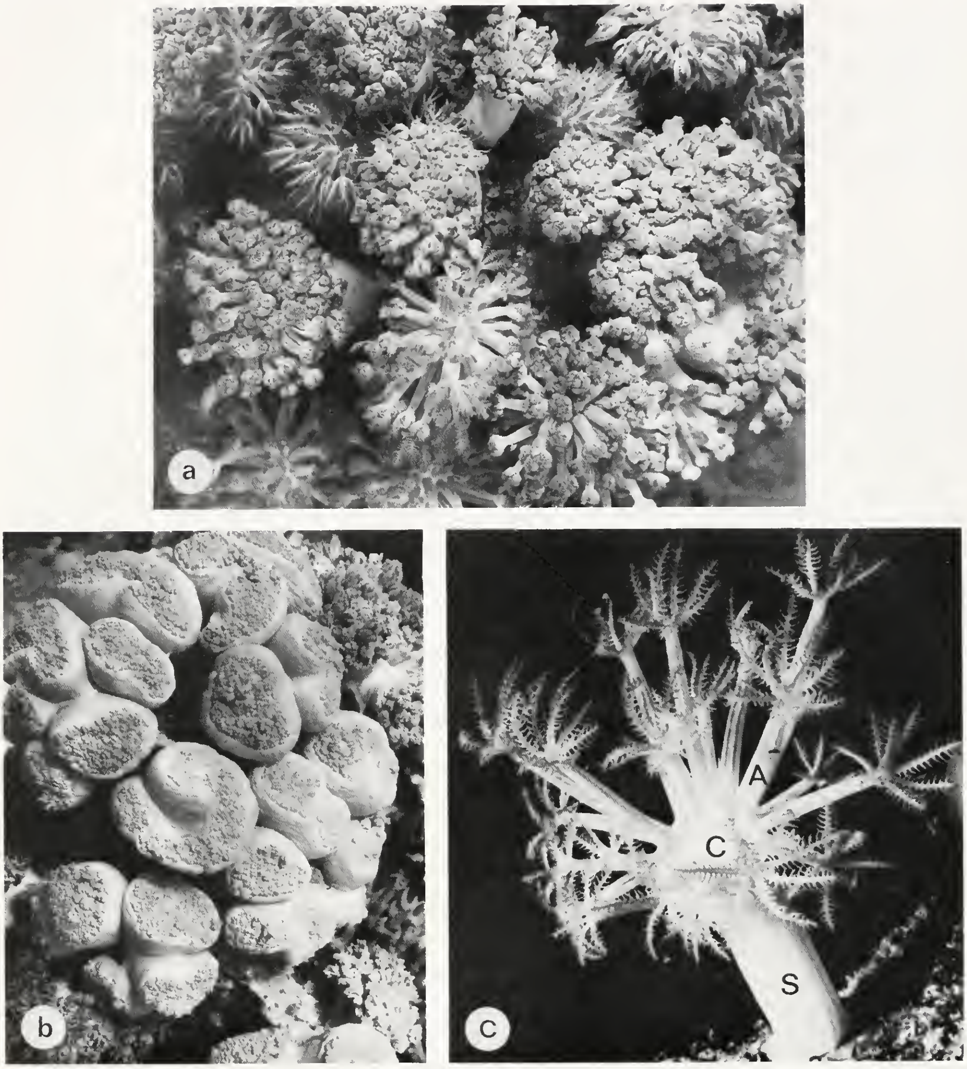

FIGURE 1. Colonies of *Xenia macrospiculata*. a: Aggregation of colonies at the coral reef of Muqebla'. b: Colonies with retracted polyps. c: Colony with extended polyps (×4). Abbreviations: A—anthocodium, C—capitulum, S—stalk.

X. macrospiculata colonies are dioecious. Hermaphroditic colonies are very rare; among more than 2000 colonies examined, only three bore both sperm sacs and oocytes. The gonads are arranged along the four lateral and two sulcal mesenteries (Fig. 2b), except for the anthocodial part of the polyps. Every year the initial gonadal primordia (IGP) start to develop at the upper part of the polyp cavity, below the anthocodium, on a small triangular projection of the septum. As maturation proceeds, more gonads develop along the edges of the mesenteries, gradually covering the surface of the mesenteries. Toward the end of gonadal development, they frequently fill most of the polyp cavities (Fig. 2c, d). The development of the IGP starts simultaneously

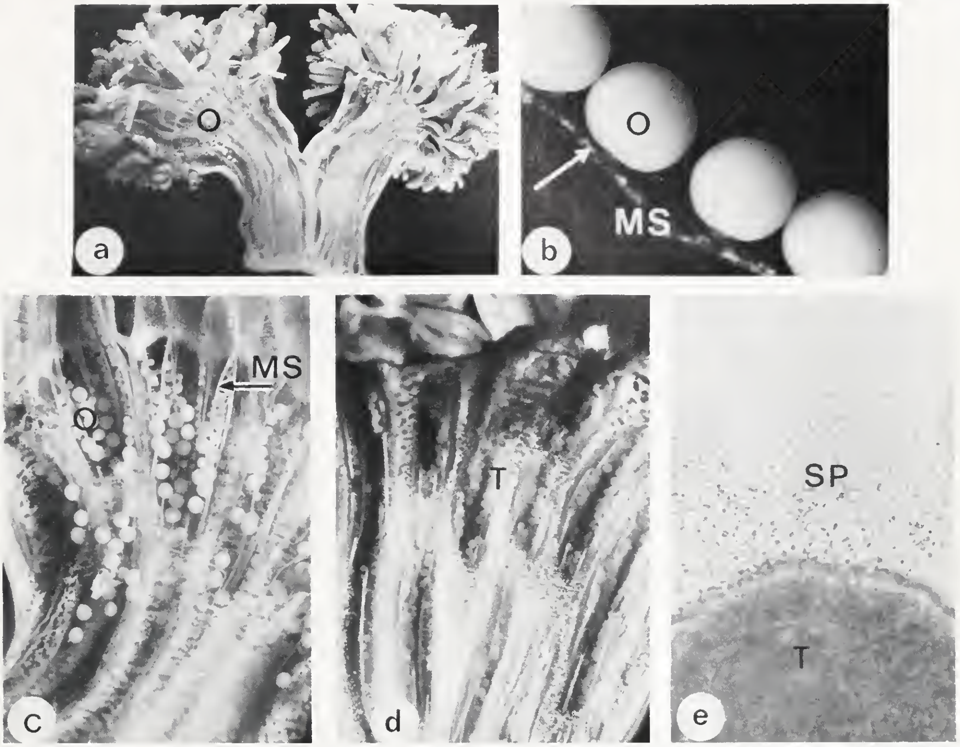

FIGURE 2. Gonads of *Xenia macrospiculata*. a: Longitudinal section through a colony showing oocytes in the syndetial part of the polyp cavities (×1). b: Oocytes arranged along a mesentery, arrow indicates the sclerites (×40). c: Oocytes in the polyp cavities of a sectioned colony (×7). d: Testes in the polyp cavities of a sectioned colony (×3). e: Mature spermatozoa burst a sperm sac. Abbreviations: MS—mesentery, O—oocyte, SP—spermatozoa, T—testes.

in all polyps of a colony. Further appearance and growth of the oocytes or the sperm sacs is completely synchronized within an individual coral.

When the diameter of the oocytes or testes is less than 30–40 μm, they can be distinguished only by histological sections. After they attain this size, sex determination is easily done by observing their color. The oocytes are spherical, opaque, and cream-white. The sperm sacs are rounded, transparent, and cream-colored. After preservation in alcohol or formalin they become yellow. Microscopic examination of the gonadal smears indicates that each oocyte contains a nucleus and a nucleolus. The sperm sacs are granulated and mature spermatozoa tend to rupture their wall (Fig. 2e). The gonads are attached to the mesenteries by short pedicels and they are surrounded by an endodermal layer rich with zooxanthellae.

Figure 3 presents the monthly reproductive state of the population during the study period in both shallow (Fig. 3a) and deep reefs (Fig. 3b). Throughout the year the majority of the colonies in each monthly sample of 20 colonies contain male or female gonads and a certain proportion of the population harbors IGP. The first IGP in the population usually start to appear in September or October and their initiation lasts a few months.

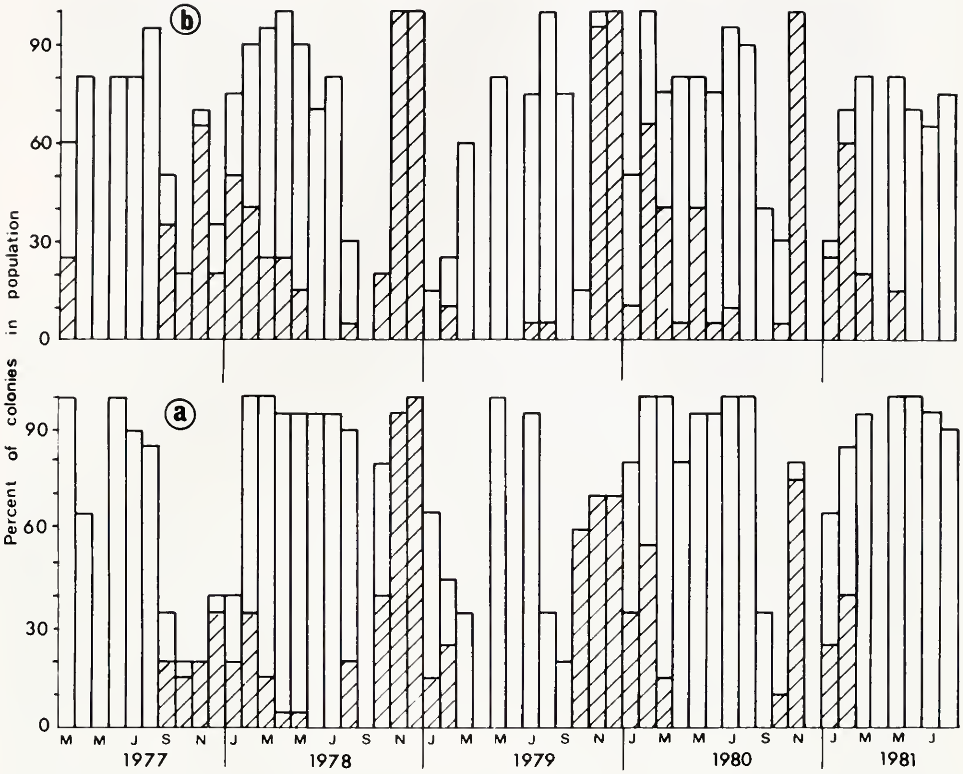

FIGURE 3. The monthly percentage of *Xenia macrospiculata* colonies with male or female gonads (blank bars) and with initial gonadal primordia—IGP (dashed bars) in 3–5 m (a) and in 27–30 m (b).

Colony size, gonadal development, and sex ratio

Figure 4 shows the size frequency distribution of *X. macrospiculata* colonies and their reproductive state in shallow water (Fig. 4a) and on the deep reef (Fig. 4b) prior to the breeding season. The colonies are divided into size groups according to their volume (cm^3). It is evident that even in the smallest size groups, gonads from both sexes are found. There is a consistent increase in the percentage of mature colonies with increasing volume in the shallow reef area, but there is a deviation from this pattern among the deep water colonies (size groups: 2.1–2.5 and 2.6–3.0 in Fig. 4b). In addition, a higher percentage of males than females is recorded in all shallow size groups (Fig. 4a) and in the majority of the deep corals (Fig. 4b). The differences between the patterns exhibited in these figures are due to local aggregations of *X. macrospiculata* composed of colonies of one sex, formed by asexual reproduction (Benayahu, 1982).

Among 624 sexually mature colonies sampled at 3–5 m, 39% were females and 61% males. At a depth of 27–30 m, out of 251 colonies, 32% were females and 68% males. These results indicate that in the population, male colonies are significantly more abundant than females (X^2, $P < 0.05$).

Annual cycle of oocyte development

The monthly changes in size-range of the oocytes are presented in Figure 5a for the shallow population and in Figure 5b for the deep one. These figures illustrate a

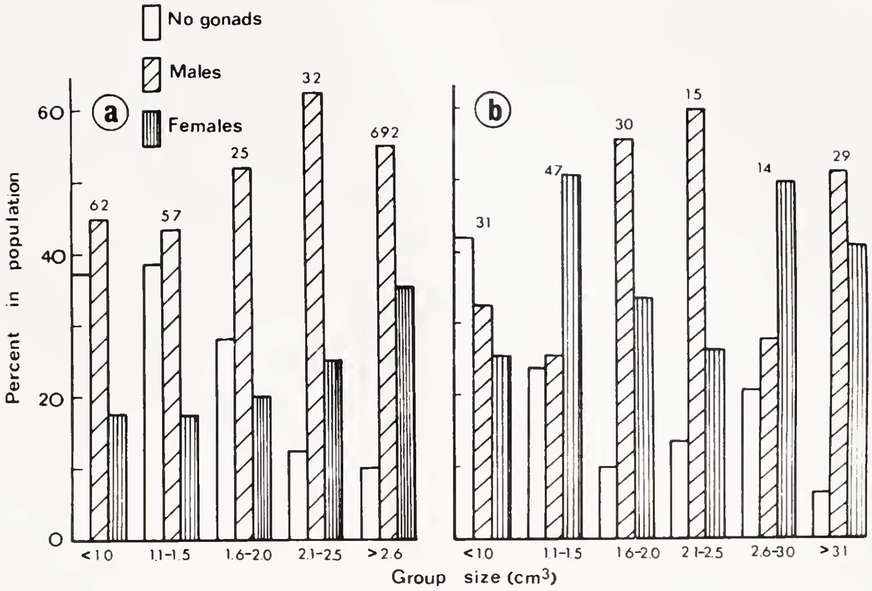

FIGURE 4. Size frequency distribution and sexual state of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b). Sample size of each group size is indicated above the bars.

similar annual pattern for both populations. The results indicate that rarely in September, but usually in October, colonies of *X. macrospiculata* bear IGP, which measure approximately 40 μm. The same narrow and low size range of oocytes is also recorded during the following months. By February, the oocytes reach a size of about 100 μm and IGP continue to develop until April–May (Fig. 5). In addition to the IGP more oocytes appear along the mesenteries for a period of 7–8 months (October–May). The prolonged oogenesis generates a wide range of oocyte sizes for several months

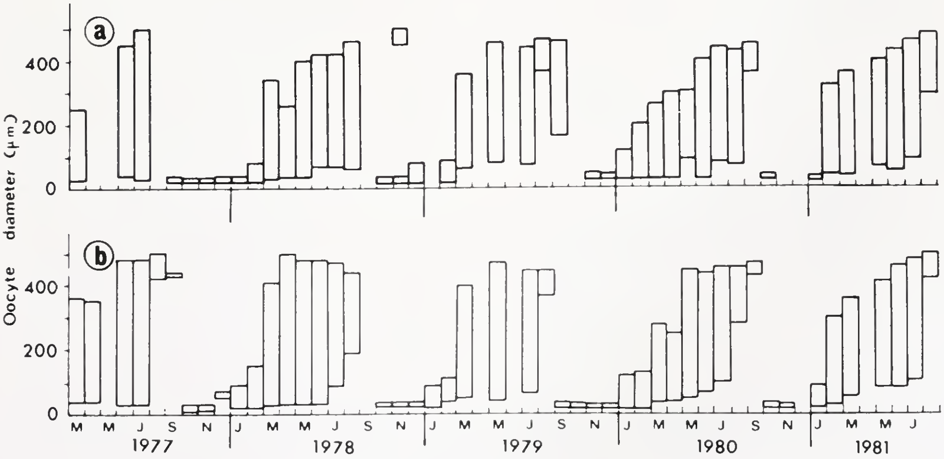

FIGURE 5. Monthly changes in the size-range of oocytes of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

during the annual cycle. In June, some of the oocytes attain their maximal diameter of approximately 450 μm. During July, August, and September the size range is still high but becomes narrower (200–500 μm). Note the result obtained in November 1978 (Fig. 5a), where the population consists of colonies with IGP and mature eggs. This is the only time when oocytes of 500 μm were found late in November.

The monthly changes in mean maximal oocyte diameter on the shallow reef is presented in Figure 6a and on the deep reef in Figure 6b. These figures indicate that a successive increase of the oocyte diameter starts yearly in October. Low standard deviations around the means were recorded at the beginning and at the end of each developmental cycle. The wide range of oocyte-diameter, usually found at the middle of each cycle (February–May), causes increased deviations around the mean values. June is the first month when oocytes of at least 400 μm are recorded and such large oocytes are found throughout the following months until September (Fig. 6). The

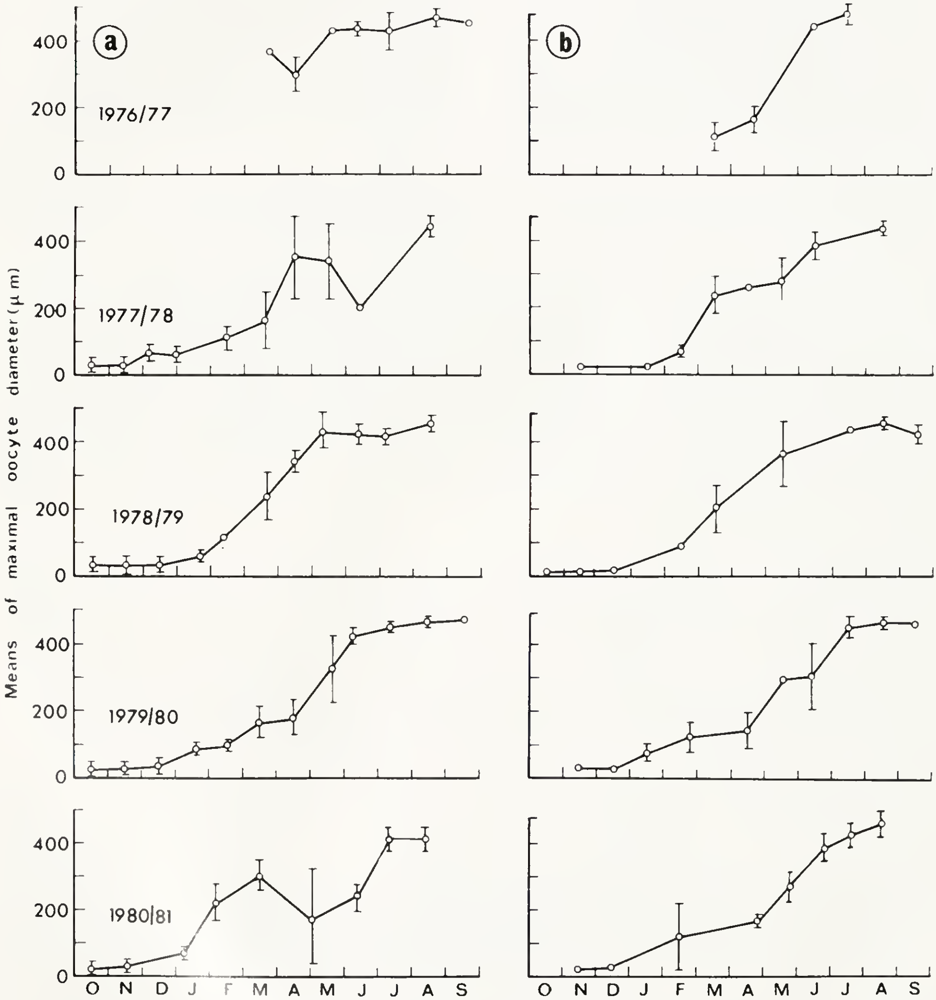

FIGURE 6. Monthly changes of the means of maximal oocytes diameter of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

largest oocytes recorded attained a diameter of 500 μm, which indicates a similar pattern of oocyte-development in the two depth zones.

The means of mesenterial areas covered by oocytes fluctuate during the annual gonadal development throughout the year (Fig. 7). A successive increase in space occupied by the oocytes along the septae is evident during October–May due to initiation of newly developed oocytes and their continued growth. Since gonadal development is not synchronized within the population, high standard deviations in mean cover of oocytes along the mesenteries were recorded during April–September. A decrease in this cover usually takes place from July, caused by transfer of ripe eggs into the brooding pouches of the colonies (see Discussion).

Annual cycle of sperm sac development

The monthly changes in the spermaries size-range are illustrated in Figures 8a and 8b for the two reefs. Sperm sac primordia start to appear in October. During the following months their diameter gradually increases. Simultaneously, as described

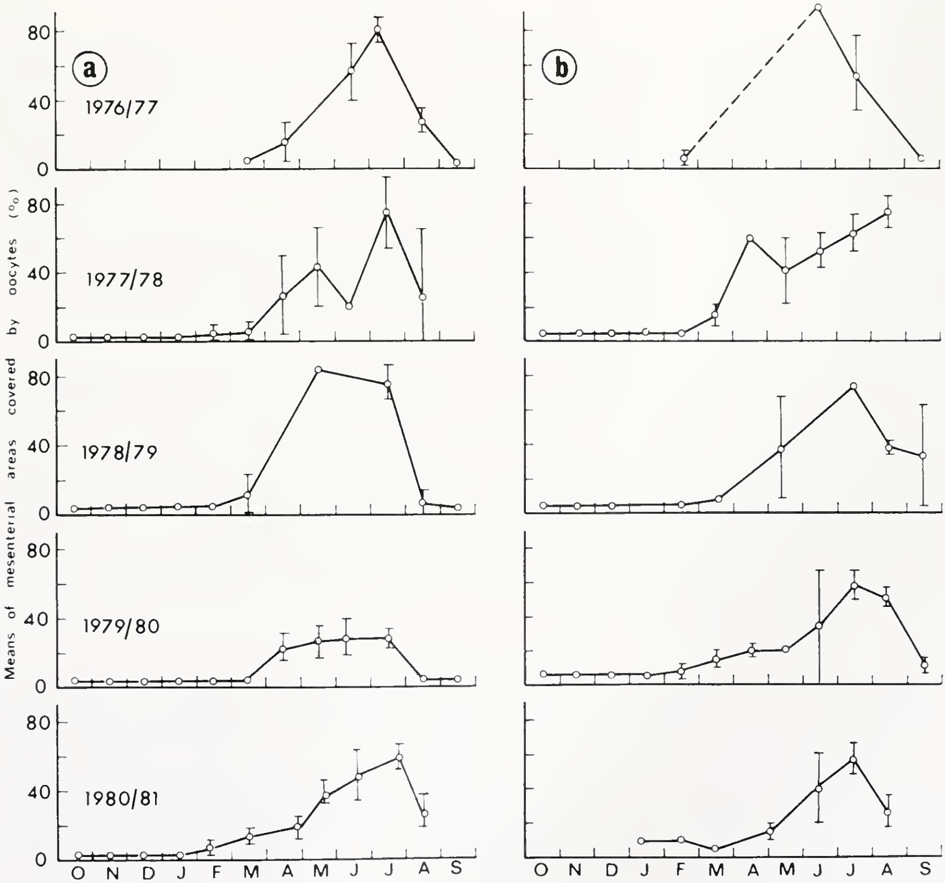

FIGURE 7. Monthly means of mesenterial areas covered by oocytes of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

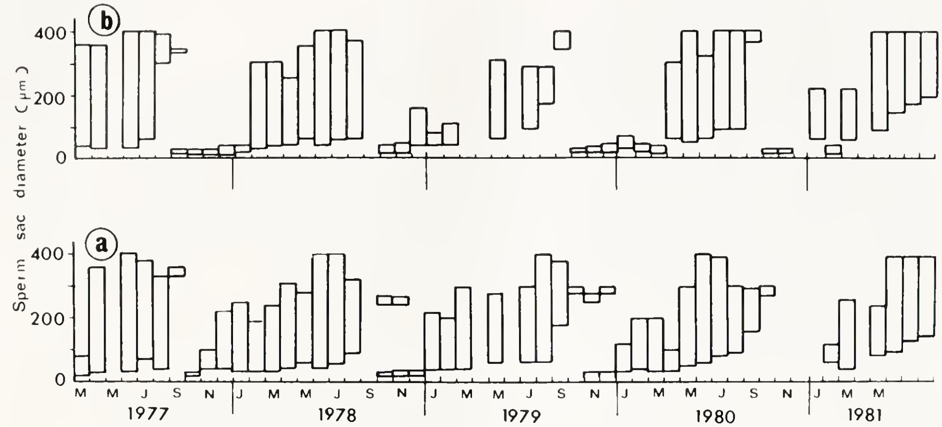

FIGURE 8. Monthly changes in the size ranges of sperm sacs of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

for the females (Fig. 5), further initiation of sperm sacs takes place in the population. In May, a wide range of sperm sac-diameter is recorded, and their size reaches a maximum value of 400 µm (Fig. 8). The size range of spermaries decreased during June–September due to the maturational process. Notice the presence of large sperm sacs in the deep-water population during October–November 1978 and November–December 1979 (Fig. 8b).

Figure 9 illustrates the monthly changes of the mean of maximal testes diameter in the shallow and deep reefs' populations. In almost every annual cycle during the first 2–5 months of development of the sperm sacs, diameters of less than 100 µm are attained. Thereafter, a faster rate of increase in size occurs until the spermaries reach their maximal size during June–July.

The means of mesenterial areas covered by sperm sacs throughout the study period are shown in Figure 10. During the first 5–6 months of each annual cycle, the space occupied by the testes usually does not exceed 10% of the septal length. The results indicate that every year during April, the surface occupation of the mesenteries by the sperm sacs accelerates rapidly, attaining a maximum of 80–90% at June–July. Towards August–September the massive spawning causes a decrease of the mesenterial areas covered by sperm sacs. The high standard deviations around the mean values (Figs. 10a, b) are due to the lack of synchronization in the development of male gonads within the population.

DISCUSSION

The present knowledge of the sexual reproduction of alcyonacean corals is mainly based on the boreal species *Alcyonium digitatum* (Hickson, 1901; Hartnoll, 1975). Among the coral reef soft-corals, *Heteroxenia fuscescens* have been studied by Gohar (1940a, b) and Gohar and Roushdy (1961). The recent studies of Yamazato and Sato (1981) on *Lobophytum crassum* and of Benayahu and Loya (1983) on *Parerythropodium fulvum fulvum,* add to the information on the life history of alcyonacean corals.

The gonadal structure of *X. macrospiculata* fits well with that of other soft corals (Ashworth, 1900; Hickson, 1901, 1931; Gohar and Roushdy, 1961). The male or

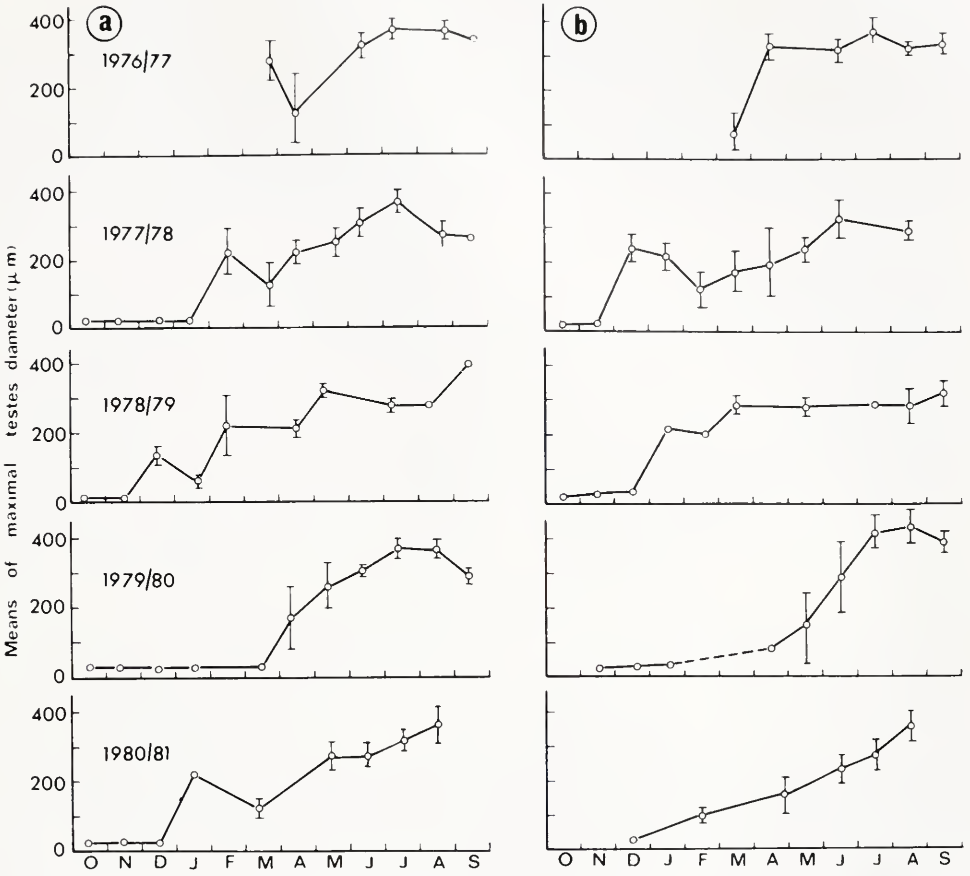

FIGURE 9. Monthly changes in the maximal means of testes diameter of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

female gonads develop along the mesenteries, originating from the endodermal tissue. While growing in size, they bulge into the syndetal part of the polyp cavity. The oocytes or the sperm sacs are attached to the mesenteries by short pedicels and surrounded by a ciliated endodermal layer. *X. macrospiculata* is a dioecious species like the majority of soft corals, although it should be noted that among the xeniids, four species are hermaphroditic: *H. elizabethae, H. fuscescens, H. ghardaqensis,* and *X. viridis* (Ashworth, 1899; Gohar 1940a).

The current study indicates the presence of male and female gonads in part of even the smallest colonies within the population (Fig. 4). Present knowledge lacks detailed information on the growth rate of *X. macrospiculata.* However, field experiments dealing with substrate colonization indicate that a linear relationship exists between colony size and age (Benayahu, 1982). The results of this study point out that colonies of about 1 cm^3 are approximately 1–2 years old. The annual maturation of the gonads in the studied species lasts at least five months (Fig. 3). Therefore, it is unlikely that a one year old colony is sexually mature. Thus, we suggest that the smallest *Xenia* colonies capable of producing gonads are approximately 2 years old. *Parerythropodium fulvum fulvum* starts to reproduce at the age of 3–4 years (Benayahu

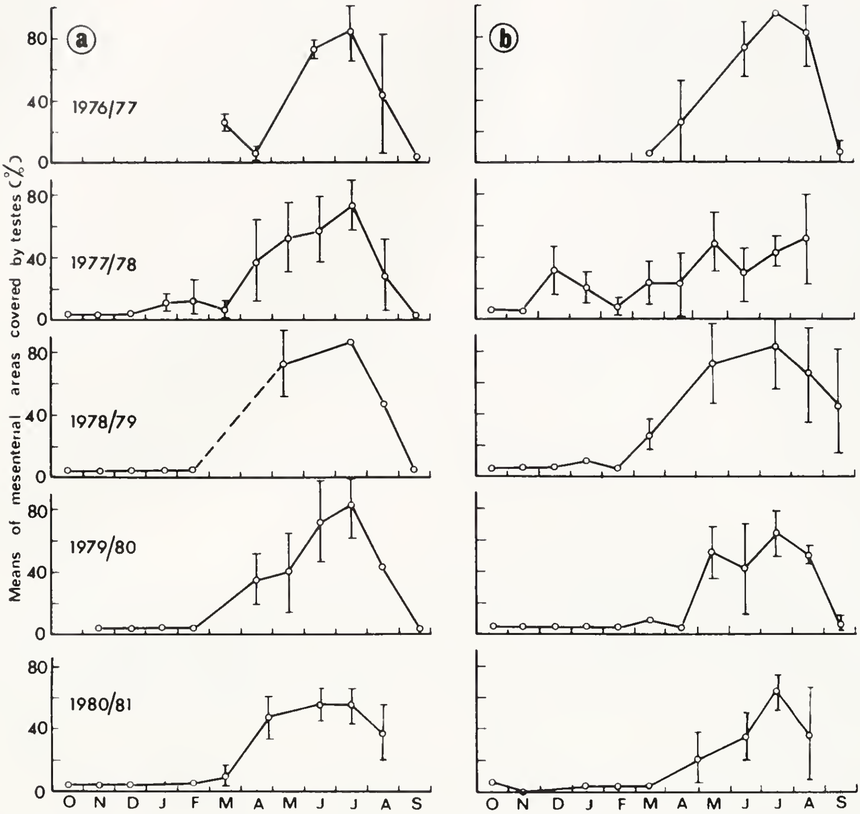

FIGURE 10. Monthly means of mesenterial areas covered by testes of *Xenia macrospiculata* in 3–5 m (a) and in 27–30 m (b).

and Loya, 1983) and *Sarcophyton glaucum* at 7–10 years (Benayahu, 1982). Reduction of age for first reproduction as exhibited by *X. macrospiculata,* suggests a shift towards high reproductive effort of the population ('r'-selection of Pianka, 1970).

The sex ratio of *X. macrospiculata* indicates a significantly higher abundance of males. We suggest that such a sex ratio is advantageous for a sessile dioecious coral such as *X. macrospiculata,* which possesses internal fertilization (Benayahu and Loya, 1984). The aggregates of *X. macrospiculata* tend to be unisexual (Benayahu, 1982), causing large distances of several meters between male and female colonies. In order to secure sperm-egg interaction, large amounts of spermatozoa are furnished by the numerous males.

Xenia macrospiculata exhibits a marked synchronization in gonadal development within the same colony. However, due to the prolonged period of gonadal initiation, the population contains colonies of various sexual stages (Figs. 3, 5, 8). Consequently, a wide range of sizes in oocytes and sperm sacs is evident almost year-round. Throughout the breeding season (May–September), the gametes gradually mature. The ripe eggs successively detach from the mesenteries, are fertilized, and pass into special brooding chambers where they develop into planulae (Benayahu and Loya, 1984). In each annual cycle, the last gonadal primordiae appear around April (Fig. 5). It is likely that these eggs ripen late and are fertilized at the end of the breeding season.

The reproductive potential of *X. macrospiculata* in terms of egg production per polyp depends on the colony size. Large colonies with long polyp cavities contain several hundred eggs in each polyp. Space for the development of oocytes and testes is used very efficiently and they tend to develop along nearly the entire length of the mesenteries (Figs. 7, 10). In each polyp young gonads develop beside older ones, thus achieving a maximal space occupation. We suggest that this feature coupled with the early age of first reproduction and the prolonged period of gonadal initiation benefit reproductive success of *X. macrospiculata.*

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LIFE HISTORY STUDIES ON THE RED SEA SOFT CORAL *XENIA MACROSPICULATA* GOHAR, 1940. II. PLANULAE SHEDDING AND POST LARVAL DEVELOPMENT

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ABSTRACT

Sexual reproduction of the soft coral *Xenia macrospiculata* Gohar, 1940 was studied for a four year period at the Gulf of Eilat (Red Sea). Ripe eggs detach successively from the mesenteries into the polyp cavities, where fertilization probably occurs. Subsequently, they pass to special brooding pouches situated near the anthocodial bases. Embryogenesis takes place in these endodermal chambers and after maturation planulae are shed via temporary openings found among the polyps. The planulation period lasts for 4–5 months (May–September) every year. Larval expulsion occurs at night in bi-weekly cycles during interlunar phases: near the first and the last moon quarters. Colony fecundity depends on its size: large colonies are estimated to produce several hundred larvae per polyp each year. Although little information exists on other planulating octocorals, the study indicates that *X. macrospiculata* exhibits a remarkably high reproductive potential, which contributes to its dominance in the Red Sea coral reefs.

Planulae of *X. macrospiculata* share common morphological features with other octocorals. The only movement exhibited by them is slow crawling over the substrate. They possess a very short planktonic phase and metamorphose into polyps immediately after settlement. These traits enable efficient colonization of available reef surfaces and hence, development of dense populations.

INTRODUCTION

Studies on the life history of Octocorallia indicate that a variety of reproductive patterns are adopted by different species (Suzuki, 1971; Goldberg and Hamilton, 1974; Grigg, 1977; Hartnoll, 1975, 1977; Weinberg and Weinberg, 1979; Yamazato and Sato, 1981; Benayahu and Loya, 1983). For a long period soft corals (order Alcyonacea) were considered to be oviparous (Hickson, 1901; Matthews, 1917). This generality was based on the study of very few species, and mainly on *Alcyonium digitatum* (Linnaeus, 1758). The early surveys concerning the xeniid soft corals did not present any information about their mode of sexual reproduction (Bourne, 1895; Ashworth, 1899, 1900; Hickson, 1931). The studies of Gohar (1940a, b) and Gohar and Roushdy (1961) pointed out for the first time that xeniids reproduce by planulae shedding. These publications concentrate on the natural history of *Heteroxenia fuscescens* (Ehrenberg, 1834) and its reproduction. In addition, Gohar (1940a) stated that *Xenia macrospiculata* reproduces by planulation. So far, nothing more is known about the dynamics of the process or development of planulae into polyps. We have

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previously described the annual cycle of gonadal development of *X. macrospiculata* (Benayahu and Loya, 1984).

The present work is based on a 4.5 year study of the abundant Red Sea soft coral *X. macrospiculata* (Benayahu and Loya, 1981). This is the first quantitative account on the dynamics of sexual reproduction of a planulating, reef-dwelling soft coral. It deals with the mode of planular brooding, the annual pattern of larval shedding, the timing of planulation, and its correlation with lunar periodicity. In addition we describe the morphological features of the planulae, their behavior, and the sequence of events leading to the development of a young colony.

MATERIALS AND METHODS

During the study on the gonadal development of *X. macrospiculata* (March, 1977–September, 1981), monthly samples were examined for planulae within the colonies (for details on study locality and sampling procedure see Benayahu and Loya, 1984). These observations indicated that larvae are found during June–September. In the present survey a quantitative examination was carried out during May–September, 1980, in order to determine the detailed reproductive state of the population throughout the breeding season. During these months the shallow water population (3–5 m) at Muqebla', located 12 km south of Eilat, was sampled 34 times. Each sample contained approximately 20 large colonies collected randomly. The colonies were carefully detached from the substrate, placed separately in plastic bags with sea water, and carried to the Marine Biological Laboratory (M.B.L.) at Eilat. Then each colony was transferred into a 1 liter aerated container, made of transparent plastic. The laboratory light regime simulated natural light conditions. This sampling procedure enabled us to continuously maintain a total number of 750 colonies during May–September, 1980 and to detect planulation at the laboratory. The containers were inspected for larvae every 7–8 hours. After planulae shedding, the colonies were removed to other containers for further observations. The planulae were counted and examined by a stereoscopic binocular microscope. A week after collection the colonies were bisected longitudinally and examined for presence of gonads or planulae (see Benayahu and Loya, 1984 for further details).

RESULTS

The planulation period and spawning

Xeniid species such as *Heteroxenia fuscescens* and *H. ghardaqensis* Gohar, 1940 often tend to expel their larvae shortly after collection from the reef, or under any other stressful conditions (pers. obs.). Such a phenomenon has never been observed among colonies of *X. macrospiculata,* despite the sampling of hundreds of corals throughout the research period. Examination of the monthly samples (Benayahu and Loya, 1984), indicates that females brood their planulae in June–September (Table I). The larvae were found inside special chambers at the upper part of the polyp cavities, near the anthocodial bases (Fig. 1a).

The continuous population sampling during May–September, 1980 indicated that the majority of the colonies bear gonads. Toward September the percent of sexual colonies decreased (Benayahu and Loya, 1984). Examination of sections of living male colonies showed that in addition to the sperm sacs attached to the mesenteries, other spermaries were free in the polyp cavities. Starting in May, the endodermal surroundings of the mature testes ruptured and mature spermatozoa were expelled. Such spermatozoa possess an elongated head, (5–7 μm), with a pointed anterior, and

TABLE I

Presence of planulae within brooding pouches of Xenia macrospiculata *during 1977–1981*

Year	June	July	August	September
1977	—	+	+	+
1978	+	+	+	—
1979	—	+	+	+
1980	+	+	+	—
1981	+	+	+	—

a tail measuring 45–50 μm. Spawning was recorded within a wide range of testis diameters (100–400 μm). during the spawning period the polyp cavities also contained many empty sperm sacs, recognizable by their wrinkled surface. Although sperm release was not observed, it is presumed that it took place through the polyp mouth.

The ripe eggs of *X. macrospiculata* detach from the mesenteries into the coelenteron, where they are probably fertilized. Then the eggs pass into special endodermal chambers (Fig. 1a), which we termed brooding pouches because larval embryogenesis occurs there. The color of the surrounding walls of the pouches is dark brown due to the high content of zooxanthellae. Each individual brooding pouch contains 15–30 embryos of the same developmental stage. Examination of colonies during the planulation period revealed that they simultaneously bear oocytes and planulae of various developmental stages.

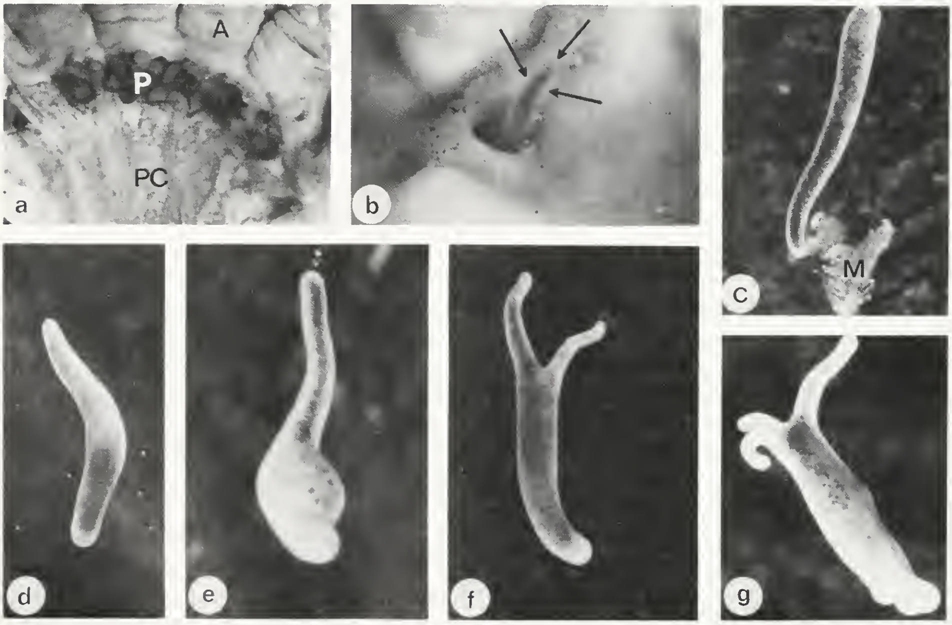

FIGURE 1. Planulae of *Xenia macrospiculata* a: Brooding pouches with young planulae (×7), anthocodium (A), planulae (P), polyp cavity (PC). b: Planula emission through a temporary opening (×15), arrows indicate the emerged larva. c: Mature planula attached by mucus (M) to the substrate (×25). d, e: Contracted planulae (×25). f, g: Malformations of planulae (×25).

Timing of planulae shedding

The close examination of 750 colonies during May–September, 1980 indicates that planulae shedding occurs with a marked periodicity. Figure 2 demonstrates the reproductive state of the population during this period. The upper part of this figure shows the dates when planulation was observed and the percent of female colonies shedding planulae during the study. The lower part of the figure represents the percent of colonies in the samples which contained planulae in their brooding pouches. These results indicate that planulation occurs twice within a lunar cycle: near the first and the last moon quarters. During each lunar quarter, planulation lasts a few days (Fig. 2). The first planulation started on 21 May and the last was observed on 21 August. The presence of some female colonies with planulae embryos at the end of August strongly suggests that the sexual reproduction continued during September. Hence, the annual reproductive period of *X. macrospiculata* lasts for 4–5 months annually (see also Table I).

Planulae shedding occurs at dusk, starting at about 1800 and becoming more intense by 2200. Within a colony this process is very rapid and it takes only a few minutes for thousands of larvae to emerge. The planulae are shed through temporary openings located among the anthocodial bases (Fig. 1b), which close immediately after planulation.

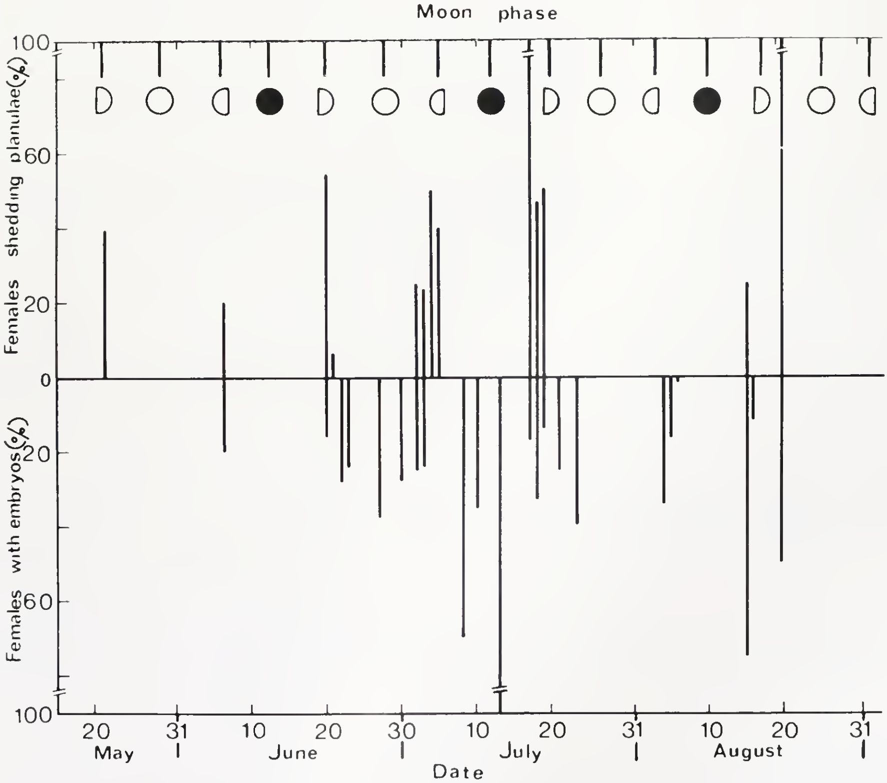

FIGURE 2. Reproductive state of female colonies of *Xenia macrospiculata* during May–August, 1980. The upper scale indicates the moon phase: D—first quarter, ○—full moon, ᗡ—last quarter, ●—new moon.

Table II presents the number of planulae emitted by seven large colonies of similar size kept at the M.B.L. The colonies were collected on 15 July and their first planulation was recorded two days later. Planulation started three days before the first lunar quarter and it continued for three successive nights. Note the low number of larvae obtained on the first night (17 July). On 21 July, the colonies were sectioned. No planulae were found in the brooding pouches, but many oocytes were attached to the mesenteries.

Figure 3 presents the number of planulae extruded by the female colonies during May–September, 1980. These results demonstrate that the number of larvae released per colony during one night is highly variable. In addition, 40% of the females did not planulate on the expected date; half of them contained immature larvae within their brooding pouches, while the other half bore only oocytes. These results point out that during the breeding season, a substantial part of the population does not shed every lunar quarter. Note that only 6% of the females released numerous larvae on a given night (several thousand per colony, Fig. 3).

Morphology and behavior of planulae

The planulae of *X. macrospiculata* are slender, reaching 2.5 mm in length and 0.15 mm in width (Fig. 1c). When fully extended they are broad at the oral end, and taper gradually toward the other end. Although their body is ciliated, planulae have never been observed swimming. Their color is cream gray, with brown spots, due to the presence of zooxanthellae. *Xenia* planulae are capable of contraction and hence of changing their body shape (Fig. 1d, e). This behavior also alters the color due to the change in the distribution of zooxanthellae. After shedding, the larvae lie vertically on the bottom of the containers, with their aboral end facing the substrate. Secreted mucus fastens the planulae to the substrate (Fig. 1c). Occasionally, the larvae change their position on the substrate by slow crawling facilitated by ciliary movement and body contractions. A negligible number of abnormal planulae (malformations) were recorded. From thousands of planulae examined only a few were the result of incomplete fusion of two or three eggs (Fig. 1f, g).

Development of the primary polyp and a young colony

Experiments dealing with substrate selection by planulae of *X. macrospiculata* (Benayahu, 1982) supply information on their metamorphosis into polyps. This study

TABLE II

Number of planulae extruded by seven colonies of Xenia macrospiculata *during successive nights in July, 1980*

Colony No.	No. of planulae			
	17 July	18 July	19 July	20–21 July
1	226	thousands	1000	0
2	3	1000	3000	0
3	10	700	350	0
4	182	thousands	60	0
5	14	0	7	0
6	262	700	12	0
7	137	thousands	thousands	0

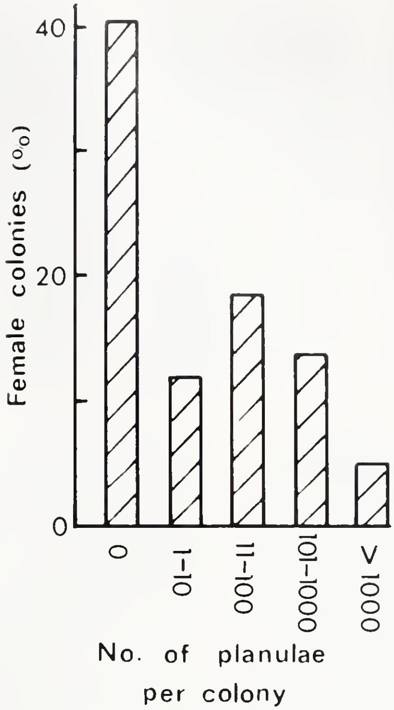

FIGURE 3. Number of planulae extruded by colonies of *Xenia macrospiculata.*

indicates that on the second day after extrusion the larvae become shorter, attaining a pear-like shape. A day later, the attached planulae become rounded, reaching ⅕ of their original length. On the 4th day, the aboral side becomes disk-like with 8–10 tentacular grooves at the oral end. On the 5th day each young polyp bears 8 primary tentacles still lacking pinnules. Later development during the 6–7th day causes unequal growth of the tentacles and the polyp body becomes elongated (Fig. 4a). During the next two days the tentacles gradually grow, achieving an even length (Fig. 4b). On the 9th day after larval emission, some tentacles bear the first pair of pinnules (Fig. 4c). Two weeks after shedding, the polyps develop four pairs of pinnules, while another two pairs are formed on the 17–18th day (Fig. 4d). One month old polyp, measures 3-5 mm and has 7–8 pairs of pinnules on each tentacle. Spicules are found at an early stage of development (Fig. 4b, c), sometimes even on the third day after shedding. Their abundance gradually increases, becoming more common on the aboral side of the tentacles.

Field experiments dealing with growth of young colonies of *X. macrospiculata* (Benayahu, 1982) indicate that budding of a second polyp occurs 3–4 months after settlement. At an age of 5–6 months a young colony bears four polyps (Fig. 4e).

DISCUSSION

Recent studies on the reproduction of planulating anthozoans are mainly concerned with sea anemones (Chia, 1976; Ottaway, 1979; Jennison, 1981), scleractinian corals (Harrigan, 1972; Stimson, 1976, 1978; Rinkevich and Loya, 1979a, b; Kojis and

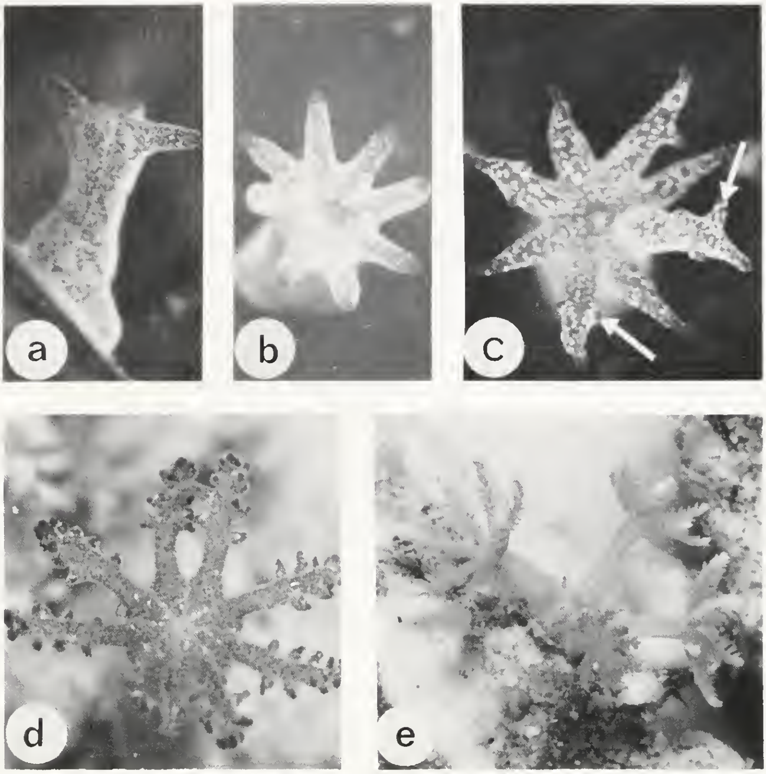

FIGURE 4. Development of a polyp and young colony of *Xenia macrospiculata*. a: 6–7 days old polyp (×8). b: 8–9 days old polyp with white sclerites (×7). c: 9–10 days old polyp, arrows indicate first pair of pinnules (×12). d: 17–18 days old polyp (×12). e: 5–6 months old colony (×1.5).

Quinn, 1981a; Moorsel, 1981; Fadlallah and Pearse, 1982, Sammarco, 1982), and gorgonians (Théodor, 1967; Kinzie, 1970; Grigg, 1977; Weinberg and Weinberg, 1979). The information gathered on the different species within each group indicates the adoption of a variety of reproductive patterns (Chia, 1976; Grigg, 1977, Kojis and Quinn, 1981a, b). Although for many years soft corals had been regarded as oviparous species, several of them were reported to reproduce through planulation (Table III). The boreal species *Alcyonium hibernicum* exhibits a parthenogenic mode of larval development (Hartnoll, 1977). The temperate soft coral *A. siderium* planulates each year for a period of one month (Sebens, 1983). The other species presented in Table III are Red Sea xeniids, reported from Gohar's study (1940a). A month-long planulation period of *X. macrospiculata* as reported by Gohar is in direct contrast to the results presented here (Fig. 2). In addition, Gohar did not find synchronization between larval shedding and lunar phase.

Kojis and Quinn (1981b) discuss the difficulties in detecting planulation among stony corals. We further suggest that larval shedding by *X. macrospiculata* exhibits several features that require a comprehensive study. We have shown that colonies of *X. macrospiculata* are dioecious, with no sexual dimorphism, and that sex ratio favors higher abundance of males (Benayahu and Loya, 1984). Further life history features presented in this paper indicate that (1) female colonies may planulate in intervals

TABLE III

Planulating soft coral species and their reproductive period

Species	Planulation period
Alcyonium hibernicum (Renouf, 1931)	July
A. siderium Verrill, 1922	August
Anthelia glauca Lamarck, 1816	no direct observation
Heteroxenia fuscesens (Ehrenberg, 1834)	March–September
H. ghardaqensis Gohar, 1940	May–June
Sympodium caeruleum Ehrenberg, 1834	no direct observation
Xenia blumi Schenk, 1896	May–June
X. hicksoni Ashworth, 1899	May–June
X. macrospiculata Gohar, 1940	October
X. umbellata Savigny, 1816	October

of two weeks or more, (2) the number of planulae extruded per colony during a particular night is highly variable, and (3) larval shedding of an individual colony lasts for only a few minutes. Under these circumstances, a long term population study examining large sample sizes is required to follow the detailed pattern of planulation.

Studies on internally brooding stony corals, sea anemones, and gorgonians indicate that planulae development occurs inside the polyp cavities (Rinkevich and Loya, 1979a; Weinberg and Weinberg, 1979; Jennison, 1981). However, among xeniids unique brood pouches function as sites for embryogenesis. We have noticed that during the reproductive season female colonies of *X. macrospiculata* may simultaneously contain oocytes and planulae of various developmental stages. Even though we lack information on the length of the planulation period of an individual colony, the present results indicate that a successive maturation of eggs occurs and that they are transferred into the brooding pouches for further development. The continuous gametogenesis of each colony (Benayahu and Loya, 1984) supports the assertion that several cycles of larval release take place every year.

The number of eggs produced by a *Xenia* colony is determined by the length of its polyp cavity (Benayahu and Loya, 1984). Even assuming that only some of the oocytes in a polyp develop into planulae, it is still probable that several hundred larvae are produced annually by each polyp. Grigg (1977) found that the gorgonian *Muricea californica* produces an average of 1.6 planulae per polyp and *M. fruticosa* 3.8 per polyp. Théodor (1967) reported a similar figure of 3–5 planulae a year for the Mediterranean gorgonian *Eunicella stricta*. The soft coral *Parerythropodium fulvum fulvum* produces 18–24 planulae per polyp each year (Benayahu and Loya, 1983). Fecundity of scleractinians is based on the number of planulae or mature eggs extruded by a coral colony at a time (Harrigan, 1972; Rinkevich and Loya, 1979b, Kojis and Quinn, 1981b). However, it is difficult to compare these results with fecundity of octocorals, since the studies on stony corals do not present the total annual planulae production of a polyp (see also Kojis and Quinn, 1981b). Nevertheless, the release of several thousand planulae by an individual colony of *X. macrospiculata* in one night (Fig. 3) indicates a markedly high fecundity. Continuous larval shedding during the reproductive season leads us to suspect that such high levels of larval production are maintained over long periods of time.

The present paper demonstrates some similarities between *X. macrospiculata* and other xeniid species studied by Gohar (1940a). The general mode of their larval development in brooding pouches and larval emission through temporary gonopores are alike among the Xeniidae. Octocoral planulae spend a relatively short period of a few hours in the water column and tend to settle on the substrate very soon after shedding (Gohar, 1940a; Weinberg and Weinberg, 1979). The motility of the planulae of *X. macrospiculata* is limited to a sluggish crawling on the substrate. Their short planktonic phase and the rapid metamorphosis into polyps support their ability to quickly colonize available space. However, these features restrict the distribution of the species and encourage the development of local aggregations of *Xenia* beds (Benayahu and Loya, 1981). In addition, *X. macrospiculata* exhibits a unique movement ability (Benayahu and Loya, 1981) and a rapid mode of asexual reproduction (Benayahu, 1982), which enable the colonies to settle successfully on the reef surface (Benayahu and Loya, in prep.). Other traits of this coral such as small body size, early age of first reproduction, and prolonged period of gametogenesis (Benayahu and Loya, 1984) benefit marked reproductive effort. Further information is needed on the life histories of other alcyonacean species, in order to compare various tactics evolved by these corals. However, the results of this study suggest that *X. macrospiculata* is an opportunistic species with a remarkable reproductive potential, causing its dominance on the Red Sea coral reefs.

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BIOLOGY OF HYDRACTINIID HYDROIDS. 1. COLONY ONTOGENY IN *HYDRACTINIA ECHINATA* (FLEMMING)

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ABSTRACT

The colonial marine hydroid, *Hydractinia echinata,* exhibits a wide range of growth morphologies during ontogeny, from sheet-like colonies of uniform ectodermal mat to colonies which produce complex networks of stolons. Colony ontogeny was quantified under uniform environmental conditions for 70 colonies of *H. echinata,* with data collected on the growth rates of the three major colony parameters: mat tissue, stolon tissue, and polyps. Analysis of the relative growth rates of the tissues clearly illustrates that variability in colony form between colonies can be attributed to continuous variation in just one of the parameters governing colony ontogeny.

INTRODUCTION

Despite their phylogenetic and ecological diversity, the many colonial phyla share a limited number of general colony forms. Much recent work has focussed on the geometrical properties and mechanical constraints of particular morphologies (Riedl, 1971; Kaufman, 1973; Wainwright, *et al.,* 1976; Alexander, 1977; Jackson, 1979; Buss, 1979; Cheetham *et al.,* 1980, 1981 and many others). Mechanical constraints clearly limit the range of physical regimes suitable for occupation by a given morphological type. For instance, highly branched upright forms may not be able to withstand areas of strong water movement, where sheet-like forms of low vertical relief may thrive; calmer waters where arborescent forms are more abundant may not be suitable for sheet-like organisms due to higher rates of sedimentation. Likewise, one can infer varying degrees of susceptibility to biological threats from geometric considerations of different morphologies. For instance, a sheet-like organism, with a large surface area in contact with the substratum, may be more susceptible to a surface-bound predator or competitor than the arborescent form. Conversely, the tree-like organism may be more susceptible to a generalized water-column-dwelling predator than the sheet-like form. Several authors have attempted, with considerable success, to predict the distribution and abundance of various colonial organisms as a function of morphological type (Wainwright and Dillon, 1969; Kaufmann, 1973; Chamberlain and Graus, 1975; Wainwright and Koehl, 1976; Brakel, 1976; Winston, 1976; Graus, *et al.,* 1977; Chamberlain, 1978; Jokiel, 1978; Buss, 1979; Foster, 1979; Jackson, 1979, and many others).

Parallelling this ecological interest in patterns of gross colony morphology, there has been an increase in interest in the phyletic distribution and fossil record of different morphologies (Boardman and Cheetham, 1973; Thomsen, 1977; Chamberlain, 1978; Van Valen, 1978; Jackson, 1979; Larwood and Taylor, 1979; Cheetham, *et al.,* 1980,

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1981; Cheetham and Thomsen, 1981; Schopf *et al.*, 1981, and many others). Jackson (1979) noted that similar morphological trends have evolved repeatedly in distantly related groups, despite considerable differences in the physiological complexity of the organisms in question. Perhaps even more striking is the observation that the same set of morphological types reappear in a given lineage following episodes of extinction. The clear ecological significance of various morphologies, coupled with the extreme conservatism of these forms throughout geologic time, suggest that detailed examination of variation in growth morphology may ultimately yield useful insights into the mechanisms of evolutionary change.

In some species, individual colonies may exhibit very different patterns in growth (Toriumi, 1955; Oliver, 1968; Boardman and Cheetham, 1973; Brakel, 1976; Buss, 1979; Jackson, 1979, and many others), often to the point that they may mistakenly be classified as separate species (Wood-Jones, 1907). Species of variable morphology are of particular interest to evolutionary studies, as these species are assumed to possess the genetic architecture necessary for the adoption of alternative morphological types. Species which are capable of producing variable growth forms have some clear advantages over those which are limited to a single mode of growth. Morphological plasticity often implies ecological plasticity, and species which exhibit variable colony morphologies consequently should be distributed over a wider range of microenvironments. Not only do morphologically variable species possess wide environmental tolerances (Jackson, 1979), but several species also to alter their morphology as a function of environmental conditions such as temperature (Crowell, 1957; Bushnell, 1966; Tusov and Davis, 1971; Jebram, 1973), microstratigraphy (Bushnell, 1966; Barnes, 1973), and nutritional regime (Crowell, 1957; Fulton, 1962; Tusov and Davis, 1971; Jebram, 1973; Winston, 1976; Jebram and Rummert, 1978).

Despite the widespread appreciation of the importance of species of variable morphology, there has been little attempt to (a) quantify this variation, (b) demonstrate a genetic basis for this variation, or (c) determine the requisite developmental shifts required to generate this variation. Here we present the results of a 'common garden' experiment in which variation in the ontogeny of colonies of the athecate hydroid, *Hydractinia echinata,* was quantified under constant environmental conditions. Our results suggest a large genetic component to the observed variability. Furthermore, analysis of rates and interactions of the major parameters of colony growth suggests that complex developmental processes and major changes in form may be largely reflected in continuous variation of a single growth parameter.

MATERIALS AND METHODS

Hydractinia echinata is an athecate colonial hydroid commonly found on gastropod shells inhabited by hermit crabs of the genus *Pagurus.* A mature colony consists of an encrusting, spinose basal mat from which several specialized zooid types arise; these include feeding polyps, reproductive polyps (blastostyles), and two specialized zooids of debatable function, the spiral zooids and the tentacular zooids. *Hydractinia echinata* is strictly dioecious and sex determination is likely under genetic control (Hauenschild, 1954). Sexual reproduction yields a planula larva which settles on a pagurid-occupied shell, metamorphoses into a feeding polyp, and then produces a colony by asexual iteration (Fig. 1).

Two different developmental processes regulate the growth of a *H. echinata* colony across a planar substratum: (1) expansion of basal mat and (2) elongation of stolons. Mat tissue is composed of a close network of entodermal gastrovascular canals surrounded by interstitial cells and covered by a uniform layer of ectoderm. Stolons are

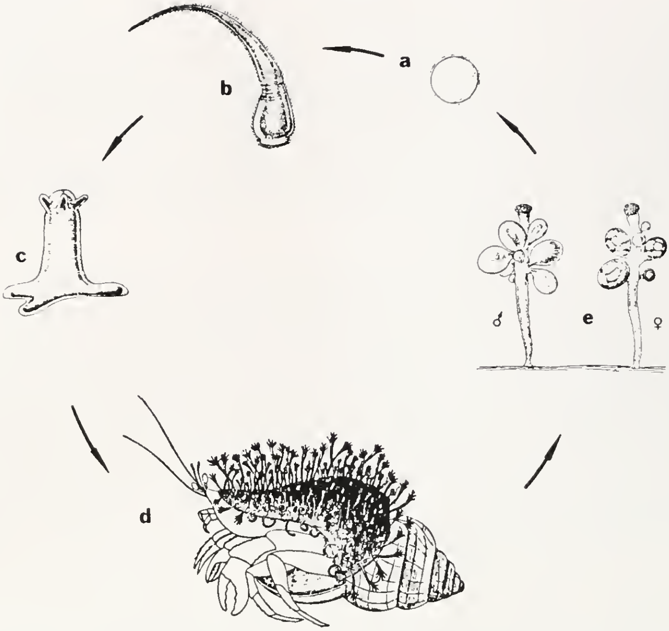


FIGURE 1. Life cycle of *Hydractinia echinata.* Fertilized egg (A) develops into crawling planuloid larva (B) which attaches to substrate and metamorphoses into a primary polyp (C). By asexual iteration this polyp develops into a mature colony (D) which will produce either male or female reproductive polyps (blastostyles) (E). (A modified from Benard-Boirard, 1962; B and C modified from Mueller, 1973; D and E modified from Hauenschild, 1954).

individual periderm-covered entodermal tubes which branch and anastomose with one another to form highly complex networks across the substrate. Feeding polyps arise from the mat tissue and, in some genotypes, from the stolon.

Although colony ontogeny has never been quantified, previous workers have recognized distinct morphological types (Schijfsma, 1939; Hauenschild, 1954). At one end of the morphological spectrum are "matty" colonies (Fig. 2A), which produce little or no stolons and grow as uniform sheets. At the opposite extreme are "viney" colonies (Fig. 2B), which produce a complex stolon network which cover the substratum much more rapidly than does a solid sheet of mat tissue. However, all viney colonies do produce some mat tissue, which eventually fills in between the pre-existing stolonal network. This difference in morphology occurs solely during colony ontogeny; on the spatially limited substrata they inhabit, all *H. echinata* colonies eventually form solid encrusting mats.

The relative rates of production of mat and stolon throughout ontogeny differ between colonies, producing a characteristic pattern in gross morphology for a given colony. We have endeavored to grow field-collected colonies under uniform environmental conditions in order to separate the genetic and environmental components of variation in growth morphology. The theory and practice of common garden experiments has been reviewed in detail elsewhere (Turesson, 1922; Clausen *et al.,* 1940). The use of field-collected colonies to assess the genetic component of variability is subject to two potential criticisms. Field-collected colonies may not be genetically uniform; genetically distinct colonies may have fused prior to collection, producing chimera individuals. This is unlikely in *H. echinata* for three reasons: (1) several

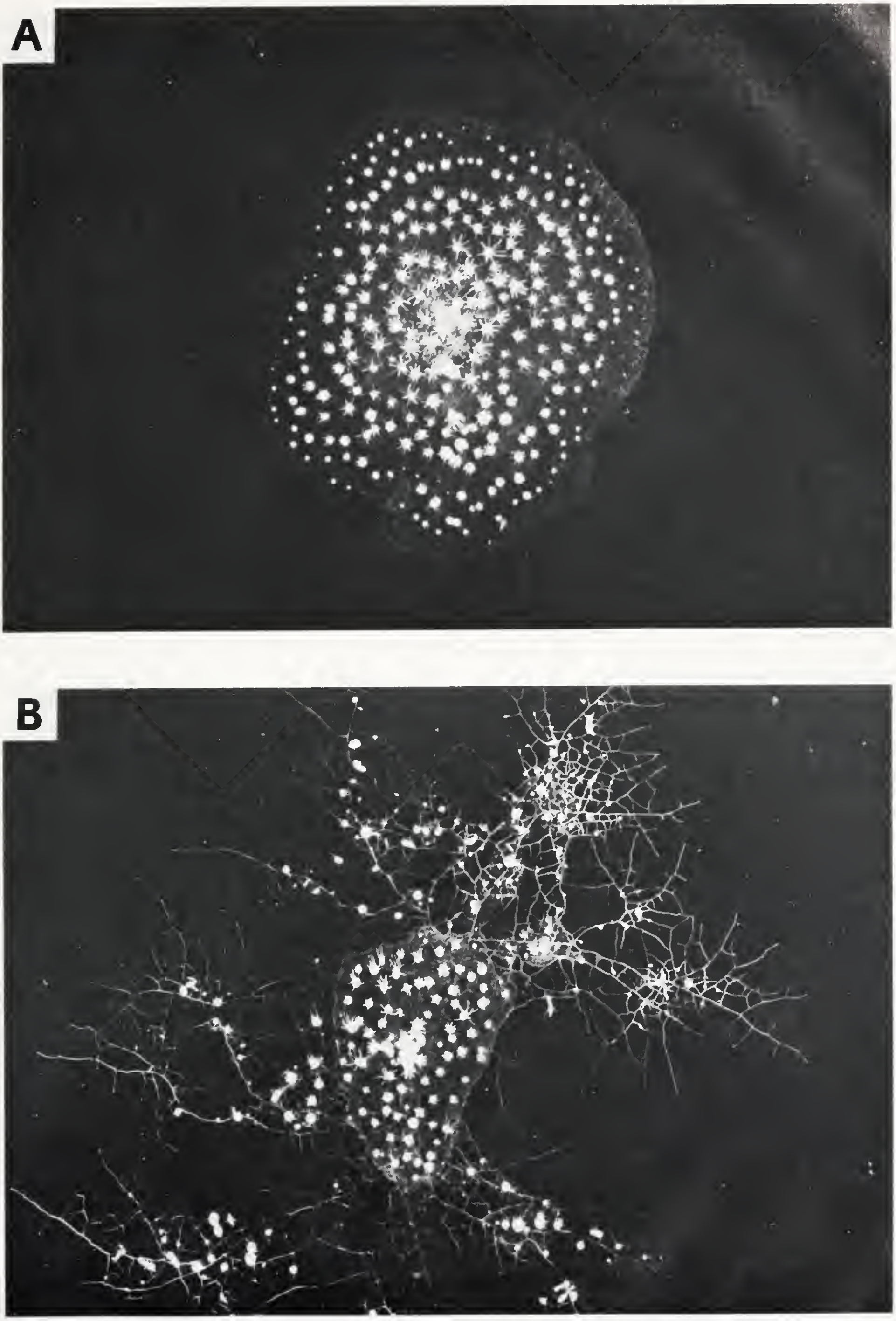


FIGURE 2. (A) A "matty" colony, which produces no stolons throughout colony ontogeny. (B) A "viney" colony, exhibiting a complex stolon network.

hundred attempted fusions between field-collected colonies have resulted in no cases of successful fusion, (2) studies of the genetics of fusibility in *H. echinata* demonstrate that fusion is limited exclusively to close kin (Hauenschild, 1954, 1956; Ivker, 1972; Buss and McFadden, unpubl. data), and (3) one-dimensional starch gel electrophoresis of 80 field-collected colonies failed to show evidence of multiple alleles for a given colony at a locus known to possess multiple alleles. The second caveat is that field-collected colonies may have been fixed in a constant morphology by some environmental factor which acted prior to the collection of the colonies. This is also unlikely for *H. echinata,* as colonies raised from planulae under constant conditions in the laboratory also display wide variability in growth morphology. Although there is no adequate substitute for quantitative genetic analysis to determine the genetic component of complex traits, the observation of wide variability in individuals cultured under constant conditions represents strong evidence for a genetic component to the observed variability.

The *H. echinata* colonies used in this study were collected from a shallow subtidal (−3 m), gravel bottom adjacent to No Man's Island, Old Quarry Harbor, Guilford, Connecticut. Using SCUBA, individuals of *Pagurus longicarpus* with hydroid-covered shells were collected haphazardly. Small pieces of basal mat containing 1–3 feeding polyps were removed from each shell with a scalpel; these explants were placed on black plexiglass culture slides, and gently held down by a loop of suture thread tied around the slide (Ivker, 1972). After 1–3 days explants had attached to slides and the threads were removed. These stock cultures were maintained in recirculating sea water at room temperature. They were fed for 2 hours daily with day-old brine shrimp nauplii, and the water changed immediately after each feeding. Food levels were sufficient to allow each colony to feed to repletion. Colonies were cleaned weekly with a small camel-hair brush to remove growth-inhibiting detritus from the surfaces of the substratum.

To initiate observations, explants of mat tissue containing three feeding polyps were excised from each of the stock colonies, and allowed to reattach to plexiglass slides in the manner described above. For each strain only one daughter colony was observed. This is justified on the basis of unpublished data which demonstrates that replicate explants removed from the same colony produce nearly identical patterns in colony ontogeny (Buss and Grosberg, in prep.). Beginning with its date of attachment to the slide, each colony was traced at approximately 4-day intervals, using a camera lucida attachment to a Wild M-5 dissecting microscope at 15×. After a period of three weeks the stolonal network of most colonies had become too extensive to conveniently trace. Thereafter, colonies were photographed at weekly intervals for another 8 weeks. To facilitate data analysis, photographs were converted to line drawings of mat area and stolons. Contact prints were placed under a dissecting microscope and traced at 7.5× using a camera lucida. These pencil drawings were then copied on a Kodak copier to yield high-contrast black-and-white representations of each drawing. Because of the time necessary to produce each drawing, not every photograph was analyzed; rather, a representative series of dates was chosen for each colony.

The cumulative growth of mat, stolon, and polyps was calculated for each colony. The number of polyps present in a colony on each date of observation was counted directly from the camera lucida tracing or contact print of the colony. The drawings of mat tissue were manually digitized on an Apple II minicomputer graphics tablet and the area of mat tissue present at each date of observation calculated. In a growing, pre-reproductive colony the mat tissue and feeding polyps are rarely resorbed, hence the area of mat tissue and the number of polyps actually present at each date of observation is equivalent to cumulative growth for both of these parameters.

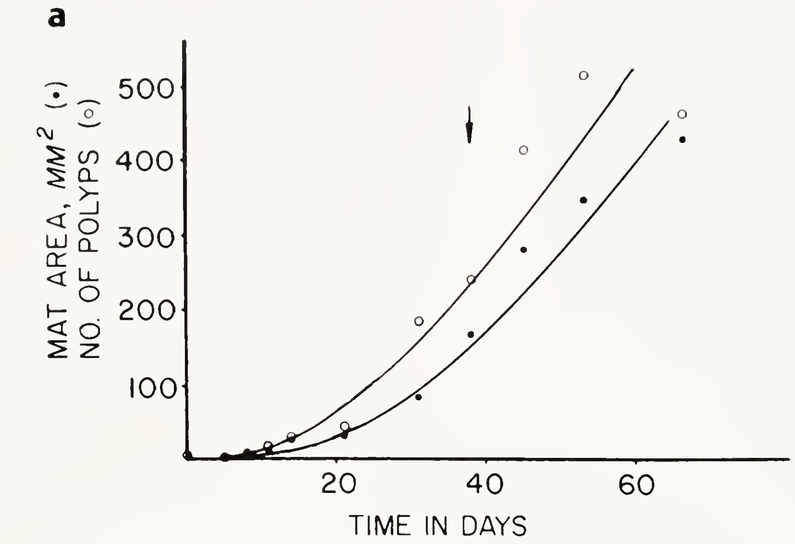
Stolonal networks were analyzed using an image analysis system (Measuronics Corp., Linear Measuring Set [LMS]) to determine total length of stolon present at each date of observation. The LMS performs an analog to digital conversion on an image which has been projected onto a video screen. The LMS is interfaced with an Apple II minicomputer and utilizes software which counts pixels to compute the desired length measurements. Complex stolon networks were processed by direct video analysis to determine total length of stolon present. Simpler stolonal networks were manually digitized using the Apple graphics tablet and the stored images were subsequently processed with the LMS to obtain stolon length measurements.

Unlike mat and polyp tissue, the length of a stolon network on any given date is not equivalent to the cumulative growth of stolon, since stolons are continually being overgrown by mat tissue. To evaluate cumulative stolonal growth, camera lucida tracings made on successive dates of observation were overlaid such that the amount of stolon which was new at each date could be determined. Only the new stolons were digitized for each of the observation dates for which camera lucida tracings had originally been made. Cumulative growth was determined by summing the values of new growth for each date. New growth was more difficult to determine from the photographic data because the magnification at which the colonies were photographed decreased as the colonies grew, thus making direct overlay of successive dates difficult. In these cases, the entire stolon network present at a given date was digitized. The photograph was then compared to earlier photos of the colony. With few exceptions, it was possible to find an earlier photograph which could be compared with the later one such that the stolon networks appeared mutually exclusive (*i.e.*, no individual stolons appeared in both photographs, yet there was also no large gap of unrecorded stolon growth between the two observations). The length of stolon present at the later date was added to the cumulative growth of stolon at the earlier date to yield cumulative growth at the later date. Although these comparisons are less reliable than direct overlays of drawings made on successive dates, any errors introduced by this process were uniformly applied to all colonies and hence do not influence comparisons between colonies.

RESULTS

Of the 72 original explants, 70 (97.3%) reattached without difficulty and grew continuously until the project was terminated. For each of these colonies (39 male and 31 female) plots were made for the cumulative growth of mat area, polyp number, and stolon length *versus* time. Plots of two of the 70 strains are presented in Figures 3A and 3B. These correspond to the 'matty' and 'viney' colonies illustrated in Figures 2A and 2B. All growth data were fitted to 1st, 2nd, and 3rd order growth equations. As may be expected with data of this sort, no single growth equation proved ideal for all 70 colonies (Kaufmann, 1981). Accordingly, comparison between colonies were made on the basis of the simplest equation, a linear regression of the log-transformed data (log [mat, stolon, polyp] = m log [time]), calculated for each growth parameter for each colony (Table 1). This approach allows one to compare a given parameter between colonies on the basis of a single statistic, the slope of a regression line with the intercept near zero (Kaufmann, 1981). The fits of the regression lines (Table I) were significant to at least the $P < 0.05$ level for mat and polyp growth for all colonies. Regressions of stolon length were significant to at least $P < 0.05$ in all but five cases. These few departures from linearity all represent colonies which exhibited a long period of no stolonal growth followed by a sudden production of stolon.

Pearson's product-moment correlation coefficients were determined to test for association between the slopes of the regression lines for each of the three parameters.



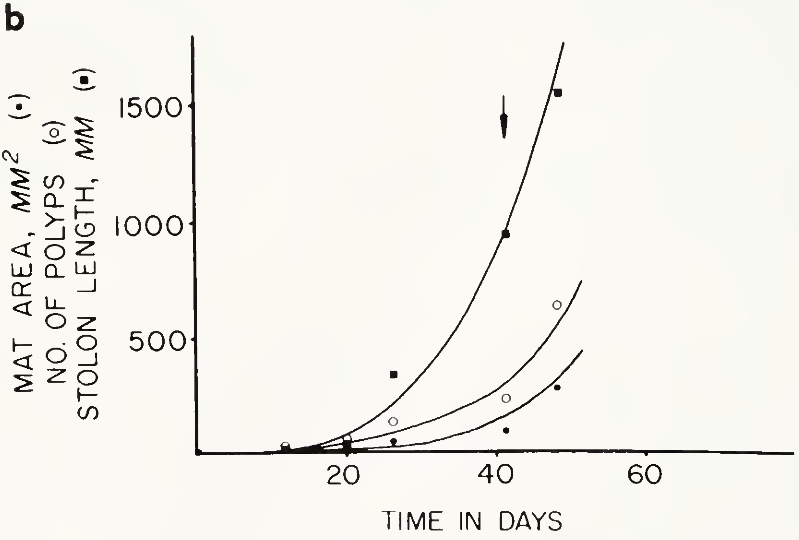


FIGURE 3. (A) Cumulative growth curves of mat tissue and polyps of colony 15, the matty colony shown in (2A). (B) Cumulative growth curves of mat tissue, polyps, and stolons of colony 141, shown in (2B). Arrows indicate date at which photograph was taken. Curves are fitted by hand.

Each slope value was weighted by the inverse of the variance of its regression to account for the differing confidence in the estimate of the slope for each growth curve. There is no significant association between the growth rates of mat and stolon ($s = -0.0559$, $P > 0.645$), nor is there any association between the growth rate of stolons and polyps ($s = 0.0839$, $P > 0.489$). There is, however, a significant association between the growth rates of mat and polyps ($s = 0.297$, $P = 0.012$). There are no significant differences between the sexes in any of the three growth parameters (polyps: F = 2.80, $P > 0.098$; mat: F = 0.67, $P > 0.415$; stolon: F = 0.03, $P > 0.085$).

A cluster analysis (SAS statistical package) was performed on the growth rates to determine if the colonies could be segregated into different groups based on the values of growth rates of mat, stolon, and polyps characterizing each colony. Two different groups are present (Table I), which are separated by a distance ratio of 0.715 (number of distances within cluster: total number of distances). These two clusters represent matty (n = 15) and viney (n = 55) colonies (Fig. 4). The growth rates of neither mat (F = 1.27, $P > 0.264$) nor polyps (F = 0.85, $P > 0.358$) differed between the two clusters. The only significant difference between these groups was in the growth rate of stolon tissue (F = 4.34, $P = 0.041$).

The ontogeny of a colony of *H. echinata* is an expression of the manner in which energy derived from feeding polyps is allocated to the production of mat and stolonal tissues. Our results demonstrate (1) that variation in colony morphology is maintained under constant culture conditions, implying a large genetic component to observed variation, (2) that the variation is unrelated to colony sex, and (3) that most variation is not a result of differential allocation of resources to both mat and stolon production, rather that it is largely the difference in the rate of growth of stolonal tissue which gives rise to the wide spectrum of colony growth morphologies exhibited by *H. echinata.*

DISCUSSION

The patterns in ecological distribution and evolutionary history of various colonial growth morphologies yield a variety of predictions as to environmental and phylogenetic distribution, but tell us little about the mechanisms which generate colony form and the relative ease or difficulty in generating changes in this morphology. The form of any colony may be dissected into a number of observable growth parameters. Each parameter, however, is the result of a complex series of morphogenetic events. Ultimately, observable growth parameters must be understood in terms of the underlying genetic organization controlling both the expression of various developmental processes and the rate at which this expression is realized.

In the absence of this information, several investigators have developed simulation models directed at determination of the extent of change necessary to generate variation in observable growth parameters. This approach in colonial invertebrates has been pioneered by Braverman (Braverman and Schrandt, 1966; Braverman, 1974). Using a series of computer simulations he demonstrated that very complex developmental patterns can be generated by the recursive application of simple, probabilistic rules of growth to an environment which is consecutively changed only by the previous application of the same rules. By specifying simple rules of growth for each of the three parameters of colony ontogeny (*i.e.*, growth of stolon, stolon branching frequency, and number of polyps), Braverman and Schrandt (1966) were able to produce a computer-generated model of a colony of *Podocoryne carnea* which roughly approximated the observed growth morphology of this hydractiniid hydroid.

Computer generated patterns which approximate actual biological patterns are of considerable interest and have been developed for a variety of other systems (*e.g.*, tree and rhizomatous plant morphology: Kamiya and Togawa, 1972; McMahon, 1975; Fisher and Honda, 1977; Niklas, 1978; Halle and Tomlinson, 1978; Bell and Tomlinson, 1980, and many others). These models illustrate that seemingly complex morphological shifts may be the result of the recursive application of a very small number of changes in basic rules. The implication is that in biological systems genes may be construed as rules which direct development and the phenotype may be

TABLE I

Hydractinia echinata *colony ontogeny*

Colony	N	Slope of mat growth curve	r^2	Signif.*	Slope of polyp growth curve	r^2	Signif.*	Slope of stolon growth curve	r^2	Signif.*	Cluster	Sex
1	8	0.996	.83	$P < .01$	0.918	.87	$P < .001$	1.038	.98	$P < .001$	2	M
3	8	1.906	.99	$P < .001$	1.579	.90	$P < .001$	0.439	.47	NS	1	M
4	10	1.821	.89	$P < .001$	1.187	.89	$P < .001$	1.238	.91	$P < .001$	2	M
5	8	1.030	.77	$P < .01$	0.851	.65	$P < .05$	1.395	.42	NS	2	M
6	9	1.588	.94	$P < .001$	1.439	.93	$P < .001$	1.783	.99	$P < .001$	2	M
7	7	1.530	.85	$P < .01$	1.561	.97	$P < .001$	0.954	.87	$P < .01$	2	M
8	7	1.549	.93	$P < .001$	1.319	.77	$P < .01$	1.920	.92	$P < .001$	2	M
9	7	1.273	.90	$P < .01$	1.231	.90	$P < .01$	1.016	.94	$P < .001$	2	M
10	9	1.887	.90	$P < .001$	1.408	.94	$P < .001$	1.323	.98	$P < .001$	2	M
11	6	1.567	.95	$P < .001$	1.000	.80	$P < .05$	0.839	.99	$P < .001$	2	M
13	11	1.582	.84	$P < .001$	1.191	.83	$P < .001$	1.678	.82	$P < .001$	1	M
14	11	1.518	.99	$P < .001$	1.307	.92	$P < .001$	0.857	.34	NS	2	M
15	11	1.538	.95	$P < .001$	1.511	.92	$P < .001$	0.00	.00	NS	1	M
16	9	1.678	.72	$P < .01$	1.413	.74	$P < .01$	2.106	.68	$P < .01$	2	M
17	12	1.155	.92	$P < .001$	0.806	.86	$P < .001$	0.669	.51	$P < .05$	1	M
18	10	1.684	.94	$P < .001$	1.206	.93	$P < .001$	1.488	.96	$P < .001$	2	M
19	11	1.702	.86	$P < .001$	1.368	.90	$P < .001$	1.731	.91	$P < .001$	2	M
20	9	1.567	.94	$P < .001$	1.225	.80	$P < .01$	2.060	.92	$P < .001$	2	M
21	7	1.428	.92	$P < .001$	1.198	.90	$P < .01$	1.490	.52	NS	2	M
24	8	1.405	.94	$P < .001$	1.098	.91	$P < .001$	1.432	.99	$P < .001$	2	M
26	9	1.475	.86	$P < .001$	1.027	.85	$P < .001$	1.218	.93	$P < .001$	2	M
27	11	2.231	.90	$P < .001$	1.559	.84	$P < .001$	1.484	.89	$P < .001$	2	M
28	11	1.482	.94	$P < .001$	1.137	.86	$P < .001$	1.587	.98	$P < .001$	2	M
29	9	1.378	.88	$P < .001$	1.066	.78	$P < .01$	0.198	.32	NS	1	M
30	7	1.994	.95	$P < .001$	1.675	.94	$P < .001$	1.977	.85	$P < .01$	2	M
31	11	1.940	.93	$P < .001$	1.532	.85	$P < .001$	2.087	.79	$P < .001$	2	M
32	3	1.380	.97	$P < .001$	1.177	.84	$P < .01$	1.123	.63	$P < .05$	2	M
33	9	1.228	.94	$P < .001$	1.035	.92	$P < .001$	0.729	.91	$P < .001$	1	M
34	10	1.412	.91	$P < .001$	1.318	.85	$P < .001$	0.00	.00	NS	1	M
35	6	1.374	.95	$P < .001$	1.064	.96	$P < .01$	0.841	.99	$P < .001$	1	M
36	10	1.404	.77	$P < .001$	1.073	.89	$P < .001$	1.601	.98	$P < .001$	2	M
37	7	1.102	.94	$P < .001$	1.049	.92	$P < .001$	1.134	.96	$P < .001$	2	M

38	9	1.260	.93	$P < .001$	1.358	.92	$P < .001$	1.968	.94	$P < .001$	2	F
40	9	1.046	.94	$P < .001$	1.042	.91	$P < .001$	0.999	.47	$P < .05$	2	F
41	9	0.946	.96	$P < .001$	0.565	.79	$P < .01$	0.00	.00	NS	1	F
42	11	1.895	.92	$P < .001$	1.383	.94	$P < .001$	1.096	.95	$P < .001$	2	F
43	10	1.329	.95	$P < .001$	1.284	.93	$P < .001$	1.369	.99	$P < .001$	2	F
101	7	1.028	.83	$P < .01$	0.494	.80	$P < .01$	0.760	.95	$P < .001$	1	F
102	9	1.704	.77	$P < .01$	1.389	.78	$P < .01$	0.872	.93	$P < .001$	2	F
103	7	1.416	.95	$p < .001$	1.049	.88	$P < .01$	1.086	.98	$P < .001$	2	F
106	8	1.251	.93	$P < .001$	1.124	.94	$P < .001$	0.933	.80	$P < .01$	2	F
107	7	1.646	.99	$P < .001$	0.879	.94	$P < .001$	1.305	.97	$P < .001$	2	F
108	8	1.616	.73	$P < .01$	1.121	.64	$P < .05$	1.640	.77	$P < .01$	2	F
110	11	1.473	.95	$P < .001$	1.079	.91	$P < .001$	0.700	.90	$P < .001$	1	F
111	8	1.450	.85	$P < .01$	1.182	.79	$P < .01$	1.165	.87	$P < .001$	2	F
112	6	1.699	.93	$P < .01$	0.404	.69	$P < .05$	0.733	.97	$P < .001$	1	F
114	6	1.784	.88	$P < .01$	1.304	.84	$P < .05$	1.702	.87	$P < .01$	2	F
115	7	1.294	.93	$P < .001$	1.245	.86	$P < .01$	1.894	.88	$P < .01$	2	F
116	10	1.692	.83	$P < .001$	1.277	.80	$P < .001$	1.333	.96	$P < .001$	2	F
117	7	1.561	.97	$P < .001$	1.225	.98	$P < .001$	1.478	.69	$P < .05$	2	F
119	5	1.125	.65	NS	1.062	.73	NS	1.936	.78	NS	2	F
121	9	1.469	.99	$P < .001$	1.041	.90	$P < .001$	0.568	.55	$P < .05$	2	F
123	6	1.097	.95	$P < .01$	0.733	.80	$P < .05$	0.00	.00	NS	1	F
125	9	1.741	.92	$P < .001$	1.682	.93	$P < .001$	1.734	.96	$P < .001$	2	F
126	9	1.709	.85	$P < .001$	0.744	.98	$P < .001$	1.650	.98	$P < .001$	2	F
127	9	1.328	.99	$P < .001$	0.769	.99	$P < .001$	1.614	.95	$P < .001$	2	F
129	6	1.495	.88	$P < .01$	1.313	.82	$P < .05$	1.619	.97	$P < .001$	2	F
130	9	1.269	.94	$P < .001$	1.283	.93	$P < .001$	1.263	.74	$P < .01$	2	F
131	10	1.608	.91	$P < .001$	1.236	.80	$P < .001$	1.551	.64	$P < .01$	2	F
133	8	0.971	.88	$P < .001$	0.922	.85	$P < .01$	1.522	.68	$P < .01$	2	M
134	11	1.528	.93	$P < .001$	1.279	.93	$P < .001$	0.00	.00	NS	1	F
135	8	0.852	.81	$P < .01$	0.932	.85	$P < .01$	1.524	.95	$P < .001$	2	F
136	5	1.536	.80	$P < .05$	1.433	.66	NS	1.644	.89	$P < .05$	2	F
138	7	1.008	.82	$P < .01$	0.923	.77	$P < .01$	1.316	.86	$P < .001$	2	F
139	6	1.516	.97	$P < .001$	1.172	.94	$P < .01$	1.277	.74	$P < .05$	2	F
140	9	1.673	.92	$P < .001$	1.108	.91	$P < .001$	1.349	.96	$P < .001$	2	F
141	8	1.564	.91	$P < .001$	1.305	.88	$P < .001$	1.886	.99	$P < .001$	2	F
A	7	1.051	.83	$P < .01$	1.071	.93	$P < .001$	1.757	.97	$P < .001$	2	M
B	7	1.283	.98	$P < .001$	1.113	.95	$P < .001$	1.215	.99	$P < .001$	2	M
D	6	1.166	.90	$P < .01$	1.254	.89	$P < .01$	1.854	.74	$P < .05$	2	F

* F-Statistic, one-tailed values, df = (1, N-1).

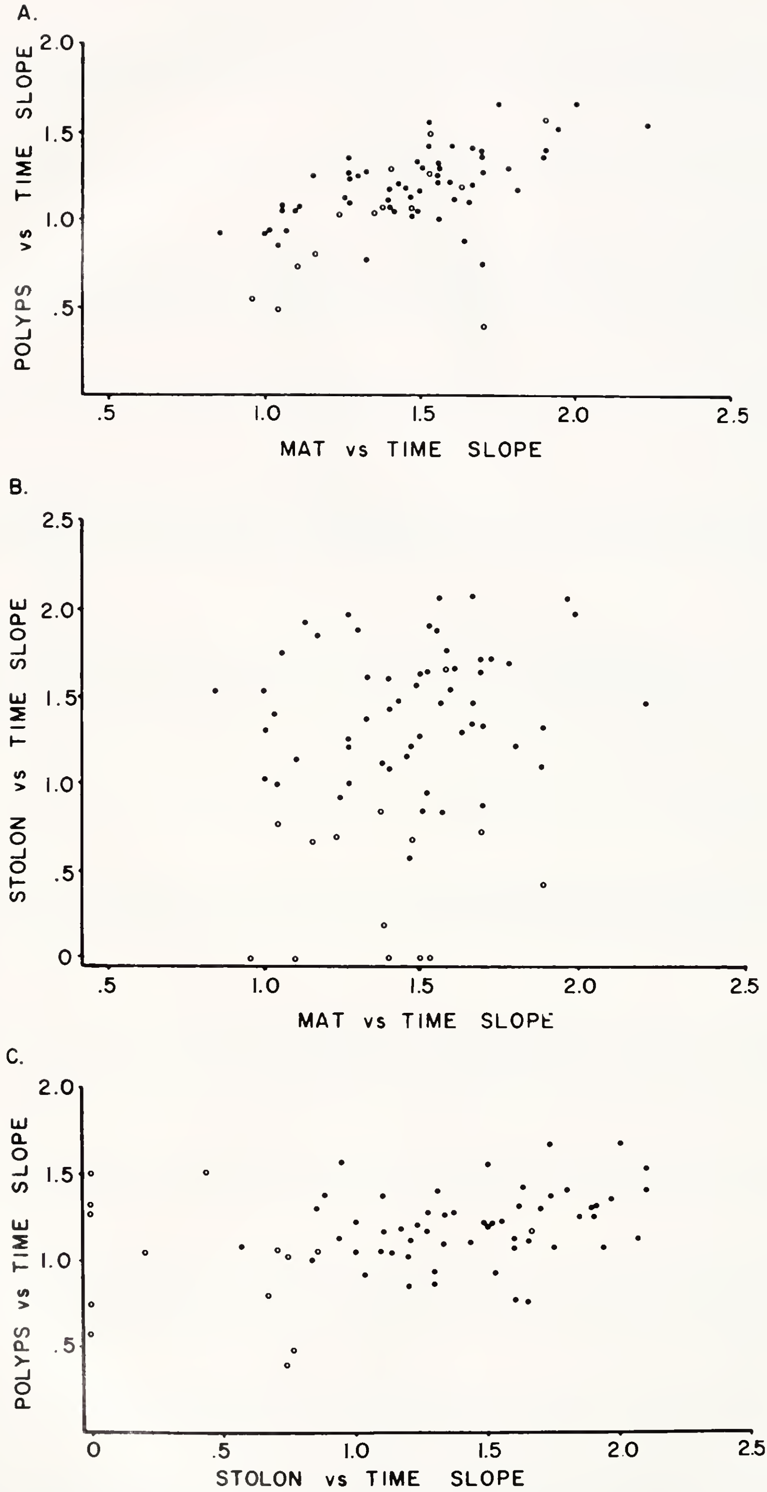


FIGURE 4. Scatter plots of the slopes of the regression lines for all 70 colonies, showing the two groups recognized by cluster analysis. (A) Mat growth rate *versus* polyp growth rate. (B) Mat growth rate *versus* stolon growth rate. (C) Stolon growth rate *versus* polyp growth rate. Open circles represent cluster 1, matty colonies. Closed circles represent cluster 2, viney colonies.

viewed as the end-product of the continuous application of a simple genetic instruction. A relatively minor change (*e.g.*, mutation or other genomic alteration) in a recursively applied rule (*e.g.*, gene) may result in a very different final product (*e.g.*, phenotype).

Although both computer modelling (Braverman and Schrandt, 1966) and geometrical treatments (Jackson, 1979) have suggested that major changes in form can be a result of variation in but a single parameter of growth, in no colonial organism with naturally-occurring variation in form has this been conclusively demonstrated. We have shown this to be the case in *H. echinata:* variation in gross colony morphology from uniform sheets to complex stolonal networks is largely controlled by variation in the rate of stolon production. Although more subtle distinctions between *H. echinata* colonies could likely be recognized by consideration of other growth parameters (*e.g.*, the branching frequency of stolons), our results clearly illustrate that large scale patterns in variability in colony form between colonies can be attributed to continuous variation in just one of the parameters governing colony ontogeny.

Models which predict that complex developmental patterns are controlled by recursive application of relatively simple rules, and that major changes in pattern consequently result from variation at a level far removed from the final phenotype are by no means tested by our data. Despite their enormous heuristic value, models of this sort are not without difficulty. Many different combinations of simple rules may yield essentially similar patterns and the actual mechanisms governing the growth of a hydroid colony can not be directly inferred from such models (Braverman and Schrandt, 1966). This problem underscores the fact that our knowledge of the manner in which eucaryote differentiation is determined has yet to have reached the stage at which the assumption of 'genes as rules' may be accepted as anything more than a working hypothesis. It is, however, this very uncertainty that makes the observation that variation in one component of the ontogenetic process has large-scale phenotypic consequences a matter of considerable interest.

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EFFECT OF TEMPERATURE ON INTERACTION BETWEEN EGGS AND SPERMATOZOA OF SEA URCHIN

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ABSTRACT

Fertilization in the sea urchin, *Anthocidaris crassispina,* showed marked temperature dependence; high temperatures (15°–30°C) were required for fertilization. In contrast, fertilization in *Hemicentrotus pulcherrimus* occurred over a wide range of temperatures (0°–30°C). The mechanism of this temperature effect in *Anthocidaris* was investigated. The number of sperm bound to the egg surface and the rate of the acrosome reaction were markedly reduced by lower temperatures (0°–10°C). Furthermore, an abnormal elongation of the sperm head tip occurred with higher frequency at lower temperatures. In contrast, the egg activation with calcium ionophore A23187 was not prevented at 10°C. The swimming activity measured by distance traveled was also relatively high at 0°C, although the activity increased as the temperature rose. These results strongly suggest that temperature exerts a direct influence on fertilization in *Anthocidaris* by acting on the acrosome reaction. The increased fertilization rate at higher temperatures in *Anthocidaris* corresponds to the higher temperature observed during the breeding season of this species.

INTRODUCTION

It is well known that development of sea urchin eggs and embryos is influenced by the temperature of the sea water. Generally, embryos develop relatively faster at high temperatures. Most sea urchin species have a specific breeding season. It is interesting that exogastrulation is induced by culture of embryos at temperatures lower than environmental condition during the breeding season (Takahashi *et al.,* 1977). Fujisawa and Amemiya (1979, 1980) also reported that adhesion of dissociated cells in sea urchin embryos is related to the environmental temperature in the breeding season. This is due, to some extent, to energy metabolism which depends on temperature. Glycogen metabolism during early development in the sea urchin, *Hemicentrotus pulcherrimus,* depends upon temperature. It has been reported that glycogen is metabolized at 15°C (Okabayashi and Nakano, 1980) but not at 20°C (Hino and Yasumasu, 1979).

In sea urchin spermatozoa, energy metabolism is influenced by temperature. We already reported (Mita and Yasumasu, 1983) that phospholipid and carbohydrate metabolism in *Hemicentrotus* sperm are activated at 20°C and only glycolysis is carried out at 0°C. Yanagisawa (1967) reported that *Hemicentrotus* sperm maintain a certain ATP level but decrease their phospholipid level after dilution at 14°C. Upon fertilization, a dramatic change in metabolism occurs in both sperm (Fujiwara *et al.,* 1983) and eggs (Yasumasu *et al.,* 1973). However, little is known about the effect of

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temperature on fertilization. In the present study, we compare the effects of temperature on fertilization, using the sea urchins, *Anthocidaris crassispina,* which breeds in summer, and *Hemicentrotus pulcherrimus,* which breeds in winter.

Materials and Methods

Preparation of gametes

Shedding of gametes of the sea urchins, *Anthocidaris* and *Hemicentrotus,* was induced by injection of 0.5 *M* KCl into the coelomic cavity. Semen was collected as "dry sperm" and kept undiluted at 4°C. Eggs were shed directly into sea water. Artificial sea water (ASW) composed of 485 m*M* NaCl, 9.6 m*M* KCl, 10 m*M* $CaCl_2$, 49 m*M* $MgSO_4$, and 10 m*M* Tris-HCl, pH 8.2 was used. The number of eggs and sperm was calculated from the protein concentration as determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. An egg and a spermatozoon contained 100 ng protein and 0.5 pg protein, respectively.

Fertilization in artificial sea water

Eggs were washed 3 times with ASW. The eggs were diluted in ASW (2×10^4 eggs/ml) at a desired temperature and then 5 μl dry sperm (3×10^8 sperm) was added to 1.0 ml egg suspension. Five min after insemination, 0.04 ml of 1% formaldehyde in ASW was dropped into the suspension. Under a light microscope, the percentage of fertilized eggs was calculated by counting the number of eggs with a fertilization membrane.

Measurement of sperm motility

Sperm motility was determined by measuring the distance in which sperm traveled through a glass capillary vessel (d = 1 mm, Vitrex, Modulohm I/S, Denmark) as described by Turner and Giles (1982) with modifications. Dry sperm were diluted in 0.5 ml ASW and a glass capillary vessel prefilled with ASW was immediately inserted into the suspension. The vessel rested at approximately 10°. At the conclusion of the incubation period, the distance which sperm traveled in the vessel was examined under a light microscope.

Oxygen consumption assay

Respiration was measured polarographically using a Clark type oxygen electrode (Yellow Spring Co., U.S.A.). Twenty-five μl of dry sperm ($2.3–2.8 \times 10^9$ sperm) were incubated in 2.5 ml ASW in a closed vessel equipped with an oxygen electrode. Oxygen consumption was determined as described by Robinson and Cooper (1970).

ATP assay

Samples were prepared for ATP assay as described by Mita and Yasumasu (1983). ATP concentration was determined enzymatically as described by Lamprecht and Trantsold (1974).

Treatment of sperm with jelly water

Jelly water was prepared by acidifying the egg suspensions with HCl to pH 5.5 for 4 to 5 min. The eggs were removed by a hand-driven centrifuge, and the supernatant was adjusted to pH 8.2 with Tris-HCl buffer. Insoluble matter was removed by centrifugation at 12,000 × *g* for 20 min. The concentration of jelly water was estimated by a carbohydrate determination using the method of Dische and Shettles (1951) with fucose as standard. Ten μl of dry sperm ($0.9–1.2 \times 10^9$ sperm) was treated with 1.0 ml of jelly water at a concentration of 20 μg fucose equivalent/ml. After 5 min incubation, the sperm were fixed by adding 1.0 ml of 4% glutaraldehyde in ASW. The percentage of sperm with the acrosome reaction was determined from electron microscopic observation (JEM 100CX, JEOL, and Hitachi HU-11-PS). Samples for scanning electron microscopy were fixed with 2% glutaraldehyde, and washed with ASW. A few drops of concentrated fixed sperm were placed onto a coverslip previously coated with 1% protamin sulfate (Nakarai Chemical Co., Japan). After 10 min, one or two drops of 4% OsO_4 were added to the droplet on the coverslip. The specimens were critical point dried in carbon dioxide and coated with gold (about 250 Å thickness) using an ion coater (IB-2 Eiko Eng'g Co., Japan). Observations were made using a scanning electron microscope (Alpha-10, Akashi Seisaku Co., Japan).

Measurement of adhesion of sperm to egg

Dejellied eggs were diluted in ASW (2×10^4 eggs/ml) at a desired temperature and dry sperm (3×10^8) were added. Vacquier (1979) reported that sperm bound to eggs from 0 to 30 s after addition of sperm to the egg suspension and that at 40 s the sperm began to detach. Therefore, after standing for 40 s the eggs were fixed with 1% glutaraldehyde in ASW. The fixed eggs were photographed under a light microscope and the binding of the sperm to the dejellied eggs was measured by counting the number of sperm bound to the periphery of the egg according to the method of Kato and Sugiyama (1978).

Artificial parthenogenesis by calcium ionophore

Eggs were washed three times with ASW and diluted in the ASW (2×10^4 eggs/ml) at a desired temperature. Calcium ionophore A23187 (Calbiochem Co., U.S.A.) was added to the egg suspension at final concentration of 50 μM. Five min after treatment, 40 μl of 1% formaldehyde in ASW was dropped into the suspension. The number of eggs with fertilization membrane was counted under a light microscope.

RESULTS

Effect of temperature on fertilization

Figure 1 shows the effect of temperature on fertilization based on formation of the fertilization membrane in sea urchin eggs. In *Anthocidaris* which breeds in summer, fertilization did not occur at 5° and 7°C. The fertilization ratio increased between 10° and 15°C, and more than 90% fertilization was obtained at temperatures between 15° and 30°C. On the other hand, fertilization in *Hemicentrotus,* which breeds in winter, did not depend on temperature and fertilization was observed even at 0°C. These data suggest that fertilization in *Anthocidaris* but not *Hemicentrotus* depends on temperature (between 0°–30°C). In order to determine how temperature affects fertilization, therefore, we investigated the effect of temperature on the interaction between eggs and sperm, mainly using *Anthocidaris.*

Effect of temperature on sperm motility

When dry sperm of *Anthocidaris* were diluted in ASW, the ATP level in sperm decreased rapidly within 5 min (Fig. 2). The constant level of ATP was higher at 0°C

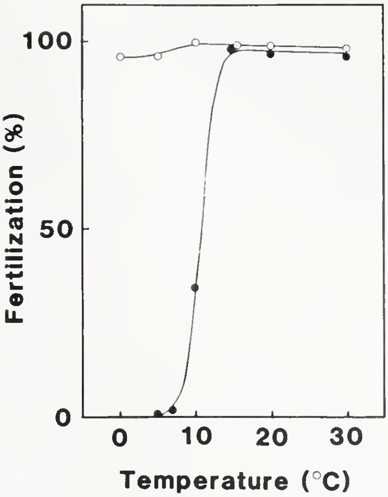

FIGURE 1. Effect of temperature on fertilization of sea urchin eggs. Eggs were inseminated by sperm and the number of eggs that formed a fertilization membrane was calculated. More than 100 eggs were observed. Values represent the mean of three separate experiments. (●): *Anthocidaris crassispina*, (○): *Hemicentrotus pulcherrimus*.

than at 20°C. ATP in sea urchin sperm is produced by phospholipid metabolism (Mohri, 1957; Mita and Yasumasu, 1983) and is consumed by their movement (Gibbons and Gibbons, 1972). Respiration is indispensable for the phospholipid me-

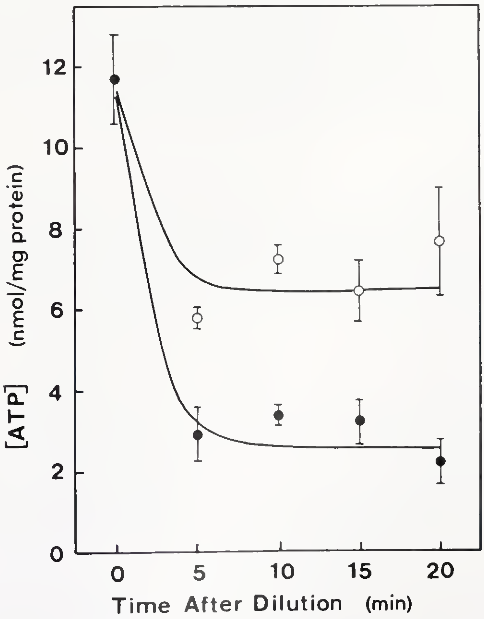

FIGURE 2. Change in the level of ATP after dilution of the dry sperm of the sea urchin, *Anthocidaris crassispina*. Dry sperm were diluted 100 fold in artificial sea water at 0°C (○) and 20°C (●). Values represent the mean of three separate experiments. Vertical bars show S.E.M.

tabolism. The respiratory rate in *Anthocidaris* sperm was correlated with temperature between 5° and 30°C. At low temperature the respiratory rate decreased markedly (Table I). These data suggest that ATP turnover is reduced at low temperatures.

Furthermore, the distance traveled by *Anthocidaris* sperm in a glass capillary vessel was correlated with temperature between 0° and 30°C (Fig. 3). The distance traveled also depends on time after dilution. The distance traveled after 10 min incubation at 20° and 30°C was almost 3 times longer than that at 0°C. However, the *Anthocidaris* sperm were motile even at 0°C. Thus, swimming activity of sperm remains at low temperatures.

Treatment of sperm with jelly water

When sperm of *Anthocidaris* as well as *Hemicentrotus* were treated with jelly water, sperm were agglutinated regardless of temperature between 0° and 30°C. Below 10°C the agglutinated *Anthocidaris* sperm were not dispersed and sperm became immotile, whereas the agglutination in *Hemicentrotus* sperm was released regardless of the temperature between 0° and 30°C. In *Anthocidaris,* the percentage of sperm with reacted acrosome (judging from filament formation) was very low between 5° and 10°C but increased above 15°C (Fig. 4). It is interesting that at low temperatures the acrosome filament is not formed but agglutination is observed.

According to scanning electron microscopic observations, *Anthocidaris* sperm with elongated head tips were more frequently observed at 4°C but they did not have the acrosomal process (Figs. 5c and d). The length of head in the elongated sperm was approximately 0.7–1.0 μm longer than that of the controls (Fig. 5a). The shape of the middle piece of sperm does change during fertilization in several invertebrate species as shown in Figure 5b (Lambert and Epel, 1979; Ikadai and Hoshi, 1981). Change in the shape of the middle piece was also observed among the elongated sperm (Fig. 5c). This may suggest that an incomplete acrosome reaction causes an increase in the number of sperm with elongated head tips at low temperatures. On the other hand, 40 to 60% of the acrosome reactions in *Hemicentrotus* sperm were constantly obtained between 0° and 30°C (Fig. 4).

Binding of sperm to eggs

Figure 6 shows that a number of sperm bound to the periphery of the dejellied eggs of *Anthocidaris* 40 s after addition of dry sperm to egg suspension. The number of bound sperm depends on temperature in the same way that the fertilization ratio was correlated with temperature (Fig. 1). Below 10°C, no spermatozoa bound to

TABLE I

Effect of temperature on oxygen consumption rate of spermatozoa of the sea urchin, Anthocidaris crassispina

Temp. (°C)	Oxygen consumption rate (nmoles O_2/min/mg protein)
5	2.60 ± 0.17
10	17.0 ± 0.52
20	66.9 ± 1.13
30	117.5 ± 0.87

Dry sperm were diluted 100 times in artificial sea water. The value is mean ± S.E.M. obtained in three separate experiments.

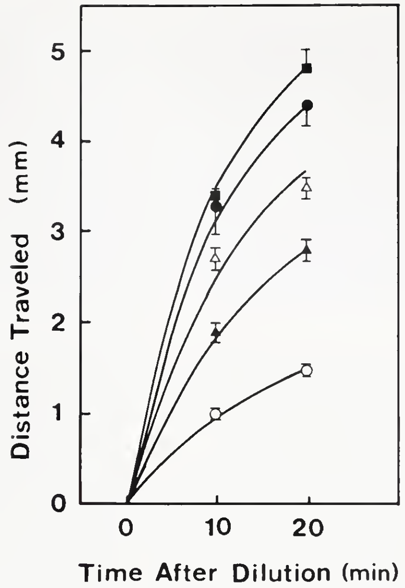

FIGURE 3. Distance traveled by the sperm of the sea urchin, *Anthocidaris crassispina*, after dilution at 0°C (○), 5°C (▲), 10°C (△), 20°C (●), and 30°C (■). Dry sperm were diluted 100 times in artificial sea water. Sperm motility was determined by measurement of the distance which sperm traveled in a glass capillary vessel (d = 1 mm). Each value represents the mean of three separate experiments. Vertical bars show S.E.M.

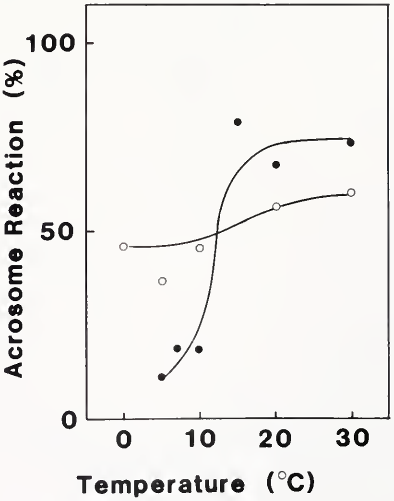

FIGURE 4. Effect of temperature on the acrosome reaction in sea urchin sperm induced by the jelly water. Dry sperm were treated with jelly water. The percentage of the acrosome reaction judging from the filament formation was monitored by electron microscopy. More than 100 sperm were observed. Values represent the mean of three separate experiments. (●): *Anthocidaris crassispina*, (○): *Hemicentrotus pulcherrimus*.

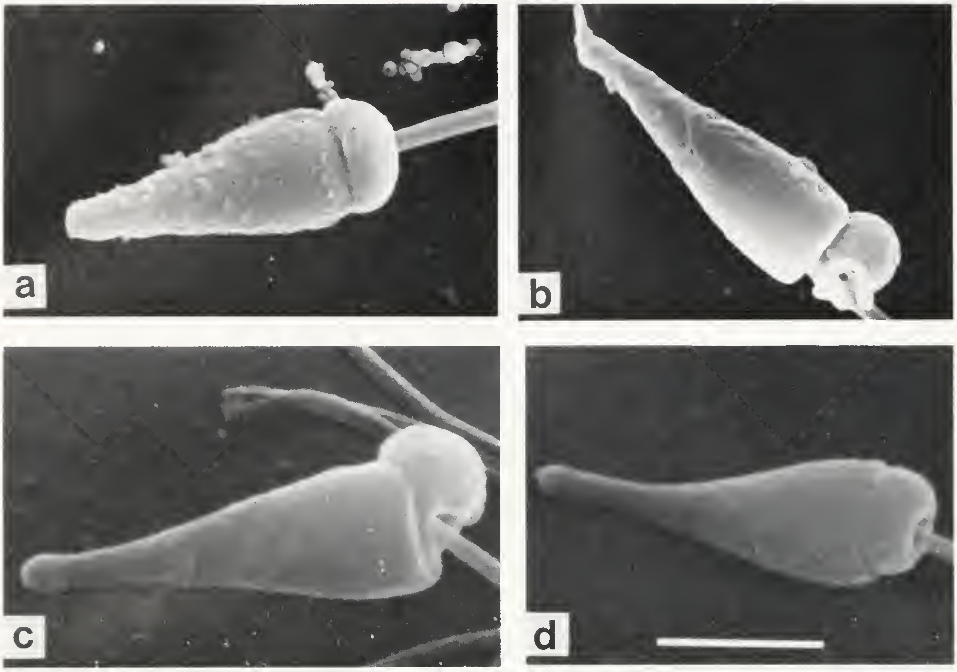

FIGURE 5. The jelly water treatment of the sea urchin sperm, *Anthocidaris crassispina* at low temperature. Dry sperm were treated with the jelly water at 4°C. (a): unreacted sperm, (b): acrosome reacted sperm, and (c) and (d): sperm with elongated head tip. Bar shows 2 μm.

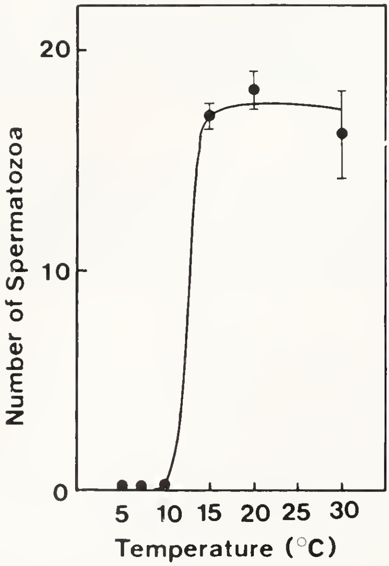

FIGURE 6. Effect of temperature on adhesion of sperm to egg of *Anthocidaris crassispina*. Dry sperm were added to the dejellied egg suspension. The binding of the sperm to the egg was measured by counting the number of sperm bound to the periphery of the egg. Each value represents the mean of five experiments. Vertical bars show S.E.M.

the egg. The number of sperm bound to the egg increased between 10° and 15°C; a constant level of bound sperm was observed above 15°C.

Egg activation with calcium ionophore A23187

Calcium ionophore A23187 activates sea urchin eggs accompanied by formation of the fertilization membrane (Steinhardt and Epel, 1974). About 40% of *Anthocidaris* eggs showed the formation of the fertilization membrane at 4°C, and more than 90% of the eggs were activated at or above 10°C (Fig. 7), after 5 min incubation with the ionophore. The ratio of the fertilization membrane formation did not change at low temperatures, even when eggs were incubated with the ionophore for more than 5 min (data not shown). On the other hand, the activation of *Hemicentrotus* eggs with the ionophore A23187 was not influenced by temperature between 0° and 30°C (Fig. 7) in the same manner that fertilization was not temperature dependent (Fig. 1).

DISCUSSION

The ambient sea water temperature during the breeding season is 0°–17°C for *Hemicentrotus* and 19°–27°C for *Anthocidaris* (Fujisawa and Amemiya, 1979, 1980). This agrees with the results in the present study that successful fertilization of *Anthocidaris* required a temperature higher than 15°C (Fig. 1). It is interesting that fertilization occurs within the range of the environmental temperature at the breeding season. However, *Anthocidaris* eggs could be activated with calcium ionophore A23187 at low temperatures (Fig. 7). This suggests that temperature does not exert a direct influence on activation. Therefore, fertilization of *Anthocidaris* is apparently regulated by the temperature dependency of sperm functions.

Energy metabolism (Fig. 2, Table I) and swimming activity (Fig. 3) in the *Anthocidaris* sperm were correlated with temperature and decreased at low temperatures. The temperature dependency in the swimming activity of sperm may explain the

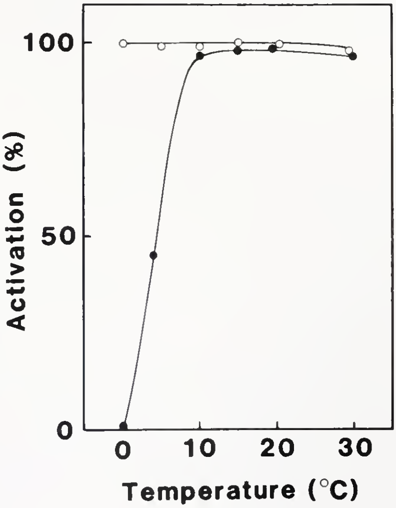

FIGURE 7. Effect of temperature on sea urchin egg activation by calcium ionophore A23187. Eggs were activated by the ionophore and the percentage of eggs with fertilization membrane was calculated. More than 100 eggs were observed. Each value represents the mean of three separate experiments. (●): *Anthocidaris crassispina*, (○): *Hemicentrotus pulcherrimus*.

failure of fertilization at low temperatures. The number of sperm bound to eggs in *Anthocidaris* was almost zero at temperatures lower than 10°C but was remarkably increased above 15°C (Fig. 6). Before adhesion of egg and spermatozoa, sea urchin sperm undergo the acrosome reaction (Dan, 1952; Afzelius and Murray, 1957). The percentage of the acrosome-reacted sperm in *Anthocidaris* also increased above 15°C (Fig. 4). These data suggest that the temperature at which the acrosome reaction occurs is closely related to successful fertilization. On the other hand, the acrosome reaction in *Hemicentrotus* sperm was not influenced by temperatures between 0° and 30°C (Fig. 4). Thus, fertilization in *Hemicentrotus* occurred at a wide range of temperatures from 0° to 30°C (Fig. 1).

There may be three reasons why the acrosome reaction in *Anthocidaris* does not occur frequently at low temperatures. First, activity of an acrosin-like enzyme which contributes to the acrosome reaction (Levine *et al.*, 1978) may depend on temperature in *Anthocidaris* but may not be influenced by temperature in *Hemicentrotus*. Second, the cell membrane of *Hemicentrotus* sperm may be more fluid at low temperatures than that of *Anthocidaris* sperm, suggesting that a low temperature condition prevents the acrosome from undergoing exocytosis. Finally, it is also possible that the polymerization of actin in the acrosomal rod is reduced under a low temperature condition. Unfortunately, little is known about these phenomena. In the present study we have reported that sperm with elongated head tips were observed at low temperature (Figs. 5c and d). The occurrence of the sperm with an elongated head tip may induce the failure of fertilization in *Anthocidaris* at low temperatures.

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SPERMATOPHORE FORMATION IN TWO INTERTIDAL CRABS *ALBUNEA SYMNISTA* AND *EMERITA ASIATICA* (DECAPODA: ANOMURA)

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ABSTRACT

Decapod crustaceans employ spermatophores in sperm transfer. Anomura spermatophores are generally pedunculate and structurally species-specific. Spermatophores of the sand crabs *Albunea symnista* and *Emerita asiatica* are macruran-type. The *A. symnista* spermatophore is non-pedunculate, and comprised of a highly convoluted tube with a firm membrane forming a cord-like mass. This spermatophoric ribbon is embedded in a gelatinous matrix. In *E. asiatica,* the spermatophores are dimorphic and pedunculate, and are attached by the peduncle-end in a row on strands of membrane. The whole spermatophoric ribbon is further embedded in a jelly-like matrix. The histology of the inner epithelial cells of the vas deferens contributing to the spermatophore mass is described. Histochemical observations on the various secretory materials and their transformation to various spermatophoric components are reported. Mucopolysaccharides are the main component. The occurrence of pedunculate spermatophores in *E. asiatica* and their absence in *A. symnista* may reflect an interesting phylogenetic interrelationship, but the epizoic attachment of spermatophores and the probable mode of dehiscence are discussed in relation to the adaptive strategies of the two crabs to the precarious intertidal zone.

INTRODUCTION

Arthropod spermatophores evolved to protect the sperm during their transfer to the females as the aquatic ancestors moved to dry land (Schaller, 1980). While most highly evolved terrestrial arthropods transfer semen by true copulation, the spermatophore is retained in many other terrestrial arthropods. Many crustaceans, though primarily aquatic, retain spermatophores. The spermatophores of decapod Crustacea can be grouped as pedunculate and non-pedunculate types (see Calman, 1909). In the former, external fertilization is typical (*e.g.,* anomurans), whereas in the brachyuran crabs internal fertilization occurs. Contrary to Brachyura and Macrura, among anomurans the spermatophores show great morphological diversity in the number of spermatophores in a ribbon, shape of the ampule, and the length of peduncle (Mouchet, 1931). In the macrurans, the spermatophore is a non-pedunculate, tubular mass embedded in a mucoid matrix that aids attachment to the female body.

Crustacean spermatophores occur in many forms, but little is known of their chemical composition. Previous authors have attributed the stabilization of spermatophore walls to chitin (Spalding, 1942; King, 1948). Recently, Malek and Bawab (1971) reported phenolic tanning in the spermatophore of the marine prawn *Penaeus*

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Abbreviations: PVD, proximal vas deferens; DVD, distal vas deferens; VD, vas deferens; AMP, acid mucopolysaccharide.

trisulcatus similar to cuticular tanning; Uma and Subramoniam (1979) did not find such hardening in the brachyuran crab *Scylla serrata*. Interestingly, in the spermatophores epizoically deposited on the female marine crustaceans, the membranous sperm sacs are invariably embedded in a thick gelatinous matrix (Berry and Heydorn, 1970). However, relatively little is known about the physiological role, if any, of these mucoid substances in sperm transport. Functional significance of such copious secretions of mucus warrants knowledge of their chemical composition.

Mole crabs of the family Hippidae deposit a macruran type of spermatophoric mass (Matthews, 1956; Subramoniam, 1977a). In the present study, two sand crabs *Emerita asiatica* and *Albunea symnista* were selected for studying the origin, chemical composition, and the strategic role of spermatophoric mass in sperm transfer.

MATERIALS AND METHODS

Emerita asiatica and *Albunea symnista* are intertidal forms found on the sandy beaches of Madras coast, India.

The testes and vas deferens in *E. asiatica* were removed by pulling the fifth leg at its base with forceps (Subramoniam, 1977a). The male reproductive system of *A. symnista* was dissected out for histological studies. The arrangement and structure of the unfixed spermatophoric mass in the two crabs were observed with bright field and phase contrast optics after vital staining. Paraffin sections of Bouins or formaldehyde fixed vas deferens were stained in Heidenhain's hematoxylin and eosin.

Histochemical properties of the spermatophoric mass were studied in paraffin and cryostat sections of vas deferens. Histochemical procedures were mainly from Pearse (1968) and Chayan *et al.* (1973). Fresh spermatophores removed from the distal vas deferens were also used. Spermatophores deposited on the female crabs were not used for histochemistry because of possible salt contamination from sea water. Mineral salts can interfere with the reactivity of complex carbohydrates (Aminoff *et al.*, 1970). Histochemical identification and characterization of the mucopolysaccharides were made by 1) periodate oxidation of *vic*-glycols, 2) detection of acid groups with basic dyes, and 3) combination of basic dye and *vic*-glycol methods (Alcian blue-PAS) for differentiating the acid mucopolysaccharides from neutral mucopolysaccharides (Spicer, 1960). Differences in the acidic characteristics of mucopolysaccharide were observed by using the basic dyes at controlled pH and at different critical electrolyte concentrations. In addition, blocking of staining by specific reactions such as methylation and acetylation were also carried out. Phenolic substances in the spermatophoric mass were located in the fresh unfixed as well as paraffin sections using ferric chloride, Millon's test, and catechol incubation techniques (Johri and Smyth, 1956; Pearse, 1968). Other histochemical tests are given at appropriate places in the text and tables.

RESULTS

Albunea symnista
Reproductive system

The male reproductive system consists of paired testes in the cephalothoracic region, and its posterior part continuing as the vas deferens (VD). This runs posteriorly for a short distance and then turns to the anterior side, forming the descending and ascending limbs (Fig. 1a). The ascending limb again turns back to open at the base of the fifth walking leg. The entire VD is divisible into a short proximal and a dilated distal portion (Fig. 1a).

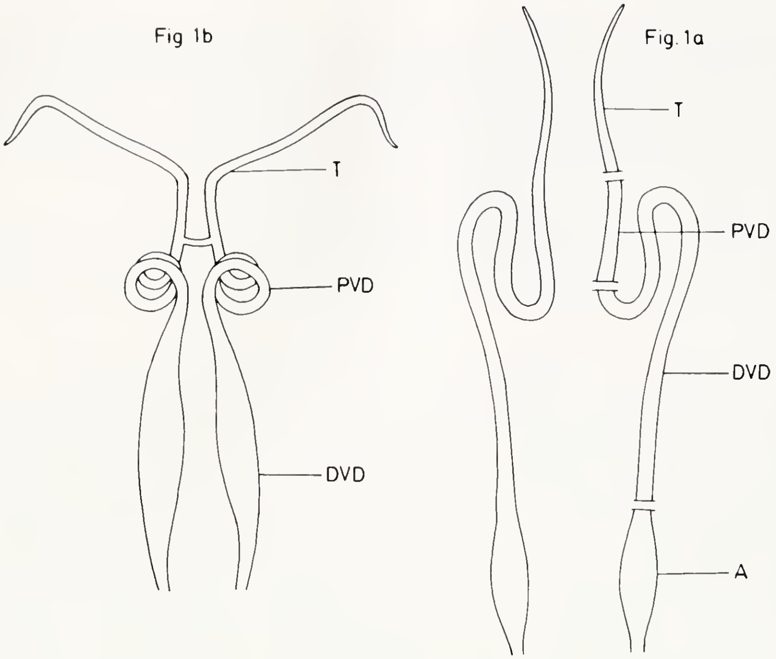


FIGURE 1. a. Diagrammatic representation of the male reproductive system of *A. symnista.* b. Diagrammatic representation of the male reproductive system of *E. asiatica.* T = testis; PVD = proximal vas deferens; DVD = distal vas deferens; A = ampulla.

Arrangement of spermatophore inside VD

The mature vesiculate spermatozoa, as released from the testes, are clumped in the anterior-most part of the proximal VD (Figs. 2, 3). This sperm mass is ensheathed in a thin wall formed by the condensation of secretory material probably emanating from the hyperactive epithelial cells (Fig. 4). This walled sperm mass passes into the enlarged distal portion of the VD. The completed spermatophore is a straight tube without accessory secretion adhering to it. As the tubular spermatophore enters the first part of the distal vas deferens (DVD), it twists and lies apposed to the ventral epithelial wall. This twisted tube, as it extends distally, becomes folded into loops and is set on to a firm membrane, forming a continuous cord in the ventral region (Figs. 5, 8). Between the loops the spermatophoric tube tends to constrict towards the ventral region and often forms node-like structures, apparently interrupting the continuity of the spermatophoric tube (Figs. 6, 7). This arrangement is regular and is found even in the extruded spermatophoric mass.

In the DVD the ventral gelatinous cord is thickened and connects the highly convoluted spermatophoric ribbon ventrally. The epithelial cells in the dorsal region of the DVD produce two typhlosole-like structures that secrete yet another material into the lumen (Fig. 9). This secretion fills up the rest of the lumen containing the spermatophoric ribbon and mixes with the secretions of the ventral epithelial cells, namely the gelatinous cord.

Histology of VD

The proximal portion is highly secretory, as evidenced by a very thick inner epithelial layer and a very thin outer connective tissue layer. The lumen is small and circular (Fig. 2). The thickness of the inner epithelial cells gradually becomes unequal; in the ventral region they are thin; in the dorsal portion they are long columnar cells (Fig. 3). The mode of secretion of these glandular cells seems to be holocrine, as evidenced by the rupture of cell membrane to release the cytoplasmic contents (Fig. 4). The hyperactive nuclei are arranged basally and the cell membranes are not distinct (Fig. 4). The epithelial cells and their secretion stain a dark blue color with hematoxylin and eosin. The released secretory material condenses into a thin membrane, which encloses the sperm mass. The dorsal glandular epithelial cells of the dilated DVD are transformed into two typhlosole-like structures whereas in the ventral region the epithelial cells are thin but highly secretory in nature (Fig. 9). The thickness of the VD wall from this region onwards results mainly from thickened multilayered outer circular and inner longitudinal muscle fibers, which probably forcibly push viscous spermatophore out. There is a bundle of connective tissue in the interior of the two typhlosoles over which there are secretory epithelial cells, producing a conical shape (Fig. 10).

The secretory material originating from the ventral epithelial cells is granular, but later coalesces to form a continuous gelatinous cord (Fig. 11). The typhlosoles and adjoining dorsal epithelial cells produce a rather less viscous fluid that is deposited in a lamellar fashion (Fig. 12). Minute granules are dispersed in this matrix. The epithelial cells are gradually reduced in thickness and the typhlosoles are also reduced in size towards the posterior part of VD (Fig. 12). In the ampullar region, the spermatophore is highly folded and the protective gelatinous matrix is very viscous (Figs. 13, 14). The spermatophore wall is very prominent and refractile, and the mature spermatozoa contained within it are vesicular in shape (Fig. 14).

Chemical composition of spermatophoric mass

The fully-formed spermatophoric mass of *A. symnista* consists of three components: the tubular spermatophore, a gelatinous cord, and the protective gelatinous matrix. Results of the histochemical analysis are given in Tables I and II. Details are given below.

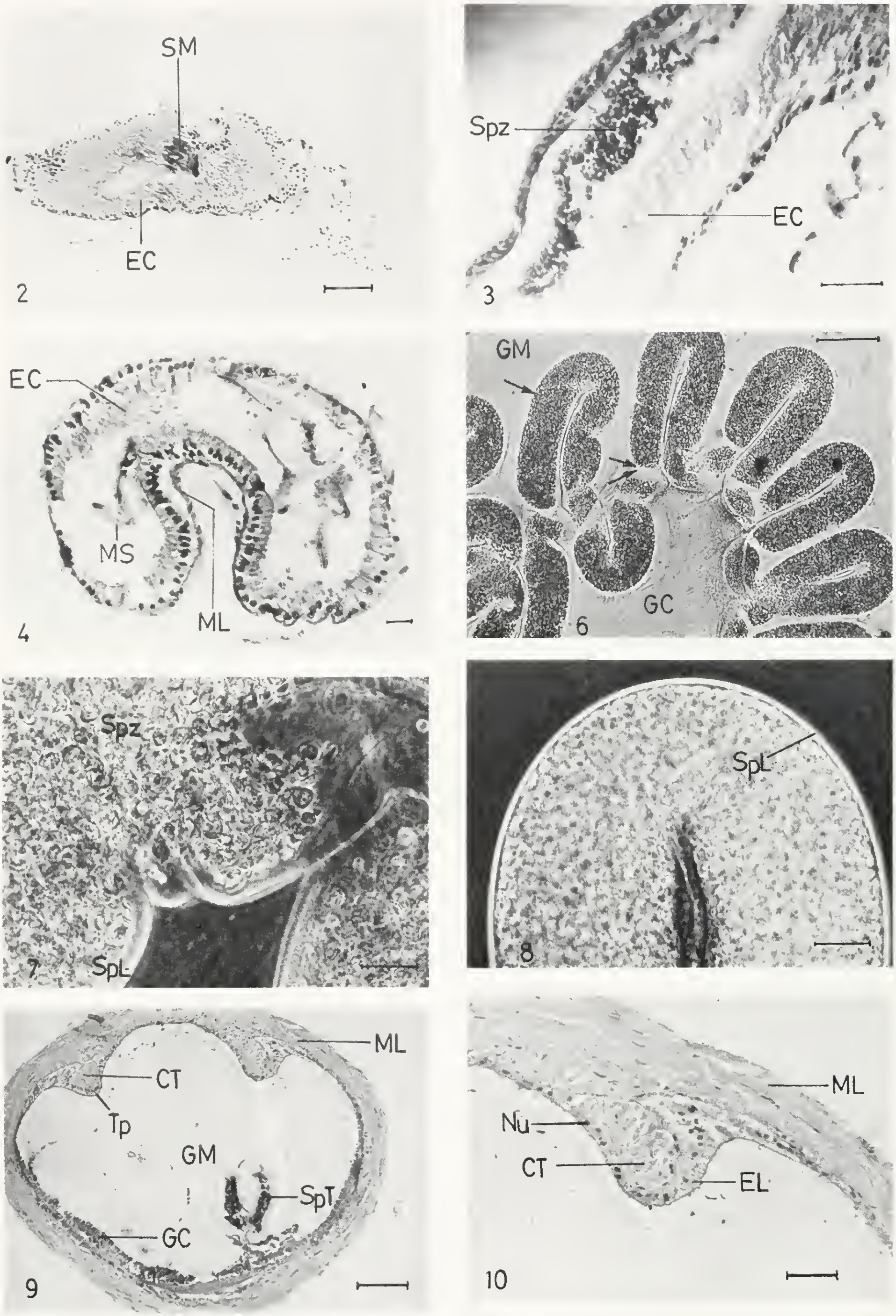
Spermatophore

The spermatophore wall is mainly composed of a neutral mucopolysaccharide, as evidenced by the PAS positivity in Alcian blue (AB) and PAS combination. With Best's carmine, this layer is a weak red color which is not abolished by diastase treatment, indicating that it is mainly mucoid in nature. Interestingly, this neutral mucopolysaccharide does not include chitin, as shown by the negative chitosan test.

The protein part of this mucopolysaccharide includes basic groups as well as tryptophanyl groups. Disulphide and sulphydryl groups are absent. Reactions to lipid tests are negative (Table I).

Sperm mass

The binding substance of the spermatozoa stains metachromaticly with toluidine blue, suggesting strongly acidic groups in the mucus. These acidic groups are in the form of sulfate polyanions, as revealed by the specific benzidine reaction of Bracco-Curti's method and the intense staining reaction with aldehyde fuchsin. However, such substances are lacking inside the spermatozoa which gave a diastase labile PAS positive reaction in the axial structure of the acrosomal vesicle. Such a differential histochemical property between the sperm mass substance and the spermatozoa is


SM
EC
2
Spz
EC
3
EC
MS
ML
4
GM
GC
6
Spz
SpL
7
SpL
8
ML
CT
Tp
GM
SpT
GC
9
ML
Nu
CT
EL
10

FIGURES 2–10. *Albunea symnista.*

FIGURE 2. Transverse section through the anterior most part of the PVD showing the thickened epithelial cells. SM = Sperm mass substance; EC = Epithelial cells. Bar = 155 μm.

Fig. 5

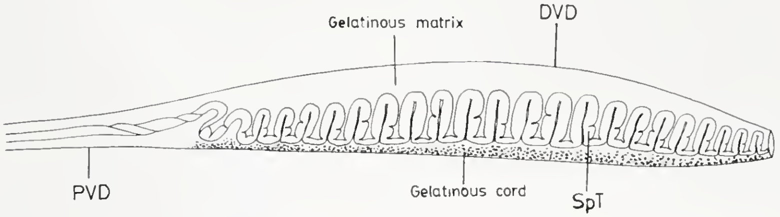


FIGURE 5. Diagrammatic representation of the part of the PVD and DVD showing the origin and arrangement of the spermatophoric mass, consisting of a convoluted spermatophoric tube (SpT), gelatinous matrix, and the ventral gelatinous cord. Note the constrictions in the spermatophoric tube in the DVD region.

clearly observed in the AB-PAS technique and with aldehyde fuchsin. Among proteins, acidic, —SH, and tryptophanyl groups are present.

Gelatinous cord

In Mallory's triple stain, the precursor granules and gelatinous cord stain red; in hematoxylin they stain blue. The gelatinous cord contains a partly diastase-digestible PAS positive substance conjugated to a protein, rich in basic and aromatic groups. A positivity to Best's carmine may indicate glycogen, but after diastase digestion the gelatinous cord continues to give a positive reaction, suggesting a mucin. Appearance of magenta with the AB-PAS technique and weak staining reaction with aldehyde fuchsin suggest neutral mucopolysaccharides. They give a positive reaction to catechol incubation indicating the enzyme phenolase. When incubated in catechol, the entire connecting cord became brown. Lipid has also been indicated by the positive Sudan black B test. Similar histochemical results were obtained for the precursor granules of the gelatinous cord.

FIGURE 3. Longitudinal section of the anterior part of the PVD showing the agglutination of spermatozoa (Spz) into a long cord. EC = Epithelial cells. Bar = 200 μm.

FIGURE 4. Transverse section of the PVD at the convoluted region showing the nature of the secretory epithelial cells (EC) surrounded by a thin muscular layer (ML); the secretory materials are found in as membranous substances (MS). Bar = 50 μm.

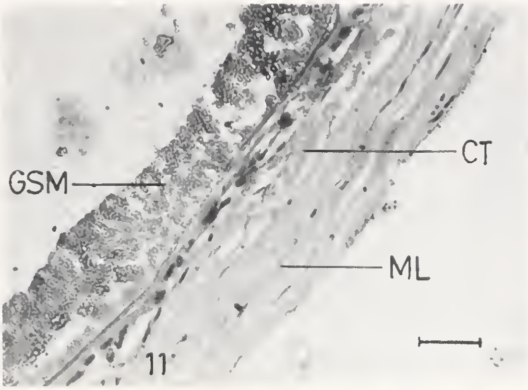
FIGURE 6. Freshly extended spermatophoric mass showing the loop-like convolution of the spermatophoric tube (↑), joined ventrally by the connecting gelatinous cord (GC) and embedded in the gelatinous matrix (GM). Note the discontinuity of the inner spermatophoric tube (↑↑) by the node formation. Bar = 10 μm.

FIGURE 7. Higher magnification of spermatophoric tube at the region of node formation under phase contrast. Spz = spermatozoa; SpL = spermatophore layer. Bar = 50 μm.

FIGURE 8. Higher magnification of the spermatophoric tube at the region of convolution under phase contrast showing the refractile nature of the spermatophore layer (SpL). Bar = 50 μm.

FIGURE 9. Transverse section of the DVD showing the two dorso-laterally placed typhlosoles (Tp), thick connective tissue (CT), and muscular layer (ML); the lumen is filled with gelatinous matrix (GM). Note the spermatophoric tube (SpT) at the ventral region, embedded on the gelatinous cord (GC). Bar = 155 μm.

FIGURE 10. Higher magnification of the typhlosole. Note the central connective tissue (CT) lined by the secretory epithelial layer (EL). Nu = nucleus; ML = Muscular layer. Bar = 60 μm.



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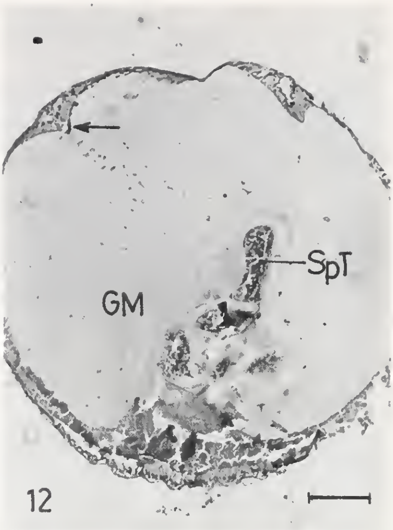

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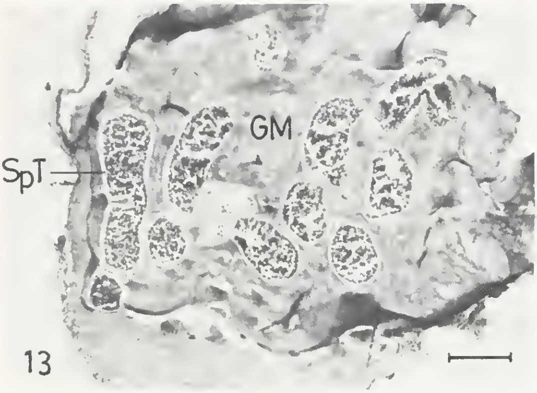

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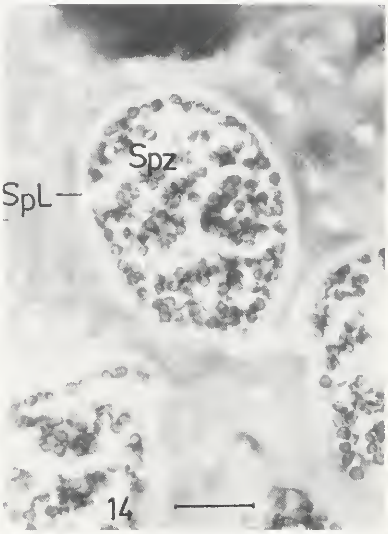

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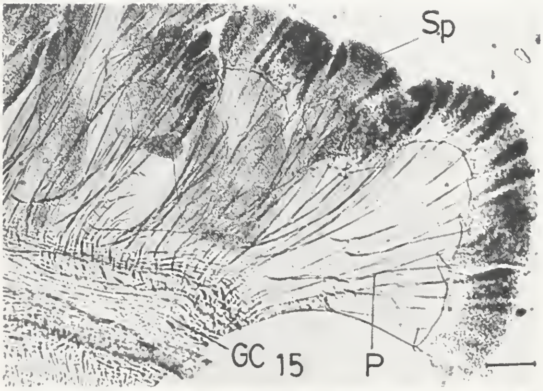

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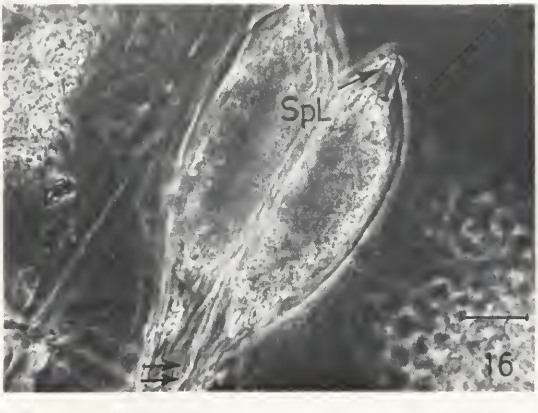

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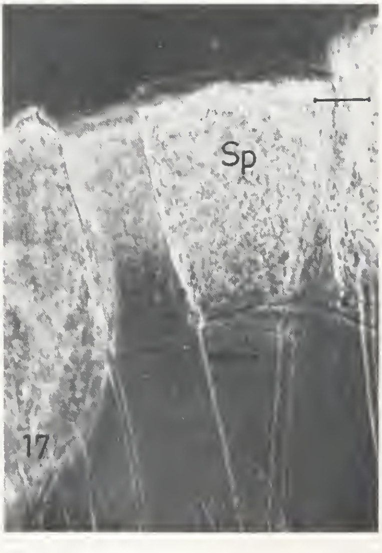

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Gelatinous matrix

The gelatinous matrix becomes blue with Mallory's triple stain and orange red with hematoxylin-eosin combination. It is metachromatic to the basic dye, toluidine blue. However, the staining intensity is greater with alcian blue than with toluidine blue (Table I). The acidic nature of mucopolysaccharide substances of the gelatinous matrix is also revealed from the AB-PAS combination. However, mild methylation did not remove basophilia obtained in the gelatinous matrix. The absence of sulphated groups is indicated by a negative reaction to Bracco-Curti's. Aldehyde fuchsin stain for differentiation of carboxyl and sulphated groups (Pearse, 1968) gives only a weak reaction. With toluidine blue the metachromatic reactions are obtained in high pH suggesting that the carboxyl groups may be predominant (Table I). However, the staining intensity with alcian blue at critical electrolyte concentration did not reveal much difference. In all the histochemical tests using alcian blue, a very strong reaction has been obtained with the gelatinous matrix.

In tests for protein, mercuric bromophenol blue gave a positive reaction. Tests for basic proteins were negative. Tests with performic acid alcian blue were positive. However this is not due to disulphide groups because the positive reaction occurred even after thioglycollate treatment. This is confirmed by the negative result of the PFAS test. Gelatinous matrix is not positive to tests for catechol, tyrosine, and other phenol containing substances, although tryptophanyl groups were detected by the DMAB test (Table II). Lipids are absent.

Emerita asiatica
Reproductive system

The vasa deferentia originate where the two limbs of the testes fuse (Fig. 1b). The VD in the proximal region is thin, convoluted, and then runs posteriorly as a straight tube gradually increasing in size.

Arrangement of spermatophores

The deposited spermatophores consist of two types of pedunculate spermatophores, one of truncated cone shape, the other of tumbler shape (Figs. 15, 16, 17). The

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FIGURES 11–14. *Albunea symnista.*

FIGURE 11. Higher magnification of the ventral wall of the DVD showing the outer thick muscular layer (ML), inner connective tissue (CT), and the concentration of granular secretory materials (GSM) forming the ventral gelatinous cord. Bar = 40 μm.

FIGURE 12. Transverse section through the distal most part of DVD showing the lamellated nature of the gelatinous matrix (GM) interspersed by granular depositions. Note the shrinking of the typhlosoles (↑) and the adjoining epithelial cells. SpT = spermatophoric tube—stained in AB-PAS. Bar = 155 μm.

FIGURE 13. Transverse section through the ampullar region showing the highly folded spermatophoric tube (spT), embedded in viscous gelatinous matrix (GM). Bar = 50 μm.

FIGURE 14. Higher magnification of the spermatophoric tube, its layer (SpL), and the vesicular spermatozoa (Spz). Bar = 40 μm.

FIGURES 15–22. *Emerita asiatica.*

FIGURE 15. Spermatophoric ribbon of *E. asiatica* showing the arrangement of the spermatophores (Sp) in the form of a ribbon with the peduncle (P) connected to the connecting gelatinous cords (GC) which, in freshly extruded condition, is fibrous. Bar = 200 μm.

FIGURE 16. Truncated shaped spermatophores of *E. asiatica.* Note the crystalline nature of the spermatophore layer (SpL) and the nipple-like projection (↑) through which the dehiscence of spermatozoa occurs. Note the drawn out peduncle (↑↑). Phase contrast. Bar = 100 μm.

FIGURE 17. Tumbler-shaped spermatophores (Sp). Note the two drawn out peduncles (↑). Phase contrast. Bar = 100 μm.

TABLE I

Histochemical characteristics of mucopolysaccharide substances and lipids of spermatophoric mass of Albunea symnista

Tests	Sperm mass*	Spermatophore wall	Gelatinous cord	Gelatinous matrix
Best's carmine (BC)	++ R	+ R	+++ R	+ R
Diastase + BC	+ R	+ R	++ R	±
Schiff alone	–	–	–	–
Periodic acid Schiff (PAS)	+ M Sperm cells	++ M	+++ M	±
Diastase + PAS	– Sperm cells	±	++	±
Acetylation + PAS	–	+ M	+ M	–
Delipidation + PAS	±	+ M	++ M	+ M
Alcian blue – PAS (AB – PAS)	++ M Sperm cells + B sperm mass substance	+++ M	+++ M	+++ B
AB – PAS after mild methylation	++ M Sperm cells + B sperm mass substance	+++ M	+++ M	+++ B
AB – PAS after strong methylation	++ M Sperm cells + M sperm mass substance	+++ M	+++ M	+++ B
Aldehyde fuchsin	+++ P	–	±	+ P
Bracco-Curti	++ BB	–	–	–
Toluidine blue at different pH				
pH 1	+++ V	–	–	+ V
pH 3	+++ V	–	–	++ V
pH 4	++ BV	–	–	++ V
pH 7	++ B	–	+ B	+++ BV

TABLE I (*Continued*)

Tests	Sperm mass*	Spermatophore wall	Gelatinous cord	Gelatinous matrix
Alcian blue: critical electrolyte concentrations of $MgCl_2$				
0.2 *M*	+	–	–	++
	B			B
0.6 *M*	±	–	–	++
				B
0.8 *M*	+	–	–	++
	B			B
1.0 *M*	++	–	–	+
	B			B
1% Aqueous alcian blue	+	–	±	+
	B			B
Chitosan	–	–	–	–
LIPID				
Sudan black B (SBB)	–	–	±	±
Delipidation + SBB	–	–	–	–
1% Nile blue	+++	–	–	++
	B			B

B = Blue; BB = Benzidine Blue; BV = Bluish violet; M = Magenta; P = Pink; R = Red; V = Violet; – = negative; ± = doubtful; + = moderately positive; ++ = positive; +++ = intensely positive; * = sperm mass refers collectively to sperm mass substance, that binds the sperm cells within the spermatophore as well as the sperm cells. When reactions are distinct for sperm cells and sperm mass substance they are indicated accordingly.

peduncles are connected ventrally to filaments, which run along the entire length of the spermatophoric ribbon (Fig. 15). The whole structure is embedded in a gelatinous matrix which binds the spermatophores tightly.

Histology of VD

The spermatophores originate in the anterior region of VD. Here, the rod-shaped mature spermatozoa are agglutinated into many clusters (Fig. 18). Each cluster of sperm is covered by a gelatinous membrane probably originating from the secretory inner columnar epithelial cells (Fig. 18). The epithelial cells are covered by a thin layer of connective tissues. A muscular layer is lacking. The epithelial layer gradually increases in thickness in the lateral sides with mid ventral and dorsal portions being atrophied into a thin layer (Fig. 19). Evidence for the formation of the peduncle from the ventral epithelium are seen in this region (Fig. 19).

In DVD, the inner epithelial cells undergo morphological changes including a shortening of the cells. In the dorsal region, they produce a typhlosole-like projection (Fig. 20). However, this differs from the typhlosoles of *A. symnista* by the absence of the inpushing of the inner connective tissues. The typhlosole secretes a frothy substance which fills the entire lumen of DVD. The ventrolateral epithelial cells secrete a gelatinous material which is deposited on the surface of epithelial cells in a manner akin to the lamellated endocuticle.

This gelatinous layer on the ventral luminal surface of the VD further condenses to form a thick cord. This branches linking itself to every one of the spermatophore

TABLE II

Histochemical characteristics of proteinaceous substances of spermatophoric mass of Albunea symnista

Tests	Sperm mass	Spermatophore wall	Gelatinous cord	Gelatinous matrix
Mercuric bromophenol blue	+ B	+ B	+ B	+ B
0.1% Aqueous bromophenol blue (ABB)	+ B	+ B	+++ B	±
Deamination + ABB	–	–	–	–
0.1% Aqueous toluidine blue (TB)	+++ V	–	–	++ V
Methylation + TB	–	–	–	–
Methylation + Demethylation + TB	+ V	–	–	+ V
Dimethylaminobenzaldehyde (DMAB)	+ B	+ B	++ B	++ B
40% Formaldehyde + DMAB	–	–	–	–
Millon's	–	–	+ BR	+ BR
Bromination + Millon's	–	–	–	–
Performic acid alcian blue (PFAB)	–	–	–	+ B
Thioglycollate + PFAB	–	–	–	+ B
Performic acid Schiff	–	–	–	–
Ferric ferricyanide (FFC)	++ PB	–	–	–
Mercuric chloride + FFC	–	–	–	–
Ferric chloride	–	–	–	–
Catechol incubation	–	–	+ Br	–
Catechol + Pretreatment with formalin	–	–	++ Br	–
Catechol + Pretreatment with alcohol	–	–	+ Br	–
TB + Light green (Anderson and Weis-Fogh, 1964)	– G	–	–	–
Methylene blue in glycerol (Anderson and Weis-Fogh, 1964)	–	–	–	–

B = Blue; BR = Brick Red; Br = Brown; G = Green; PB = Prussian Blue; V = Violet.
– = negative; ± = doubtful; + = moderately positive; ++ = positive; +++ = intensely positive.

ampules, which lie distributed on the dorso-lateral periphery of the lumen, providing, besides, an outer covering for them (Figs. 21, 22). In the distal region, the epithelial cells including typhlosole are greatly reduced.

The histochemistry of spermatophoric components

Histochemically the spermatophoric mass in *E. asiatica* is similar to *A. symnista* (Table III, IV). The sperm mass substance is composed of highly sulphated acidic mucopolysaccharides, whereas the inner layer of the spermatophore contains carboxylated AMP. In contrast, the ventral gelatinous cord, peduncle, and the outer layer of spermatophore ampullae when inside the VD, are PAS-positive. The entire gelatinous matrix stains blue in AB-PAS indicating its acidic nature. The gelatinous matrix also contains vicinyl hydroxyl groups as revealed by a PAS positivity, when used alone. Further, the acidic group of AMP is carboxylic in nature. The outer layer of the spermatophore in the freshly extruded condition is refractile to all stains. The spermatophoric mass of *E. asiatica* does not undergo 'hardening' on exposure to sea water.

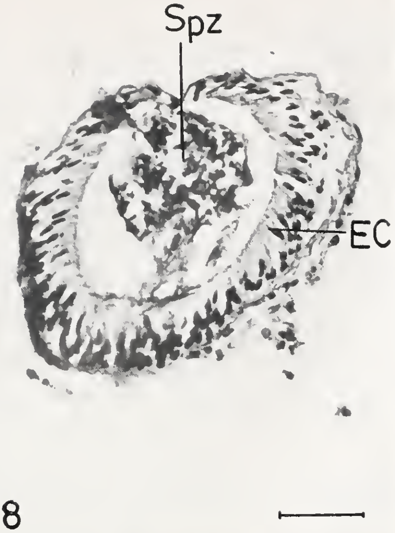
The basal gelatinous layer is highly eosinophilic, but the stalk arising from it stains blue with hematoxylin. This change in the staining reaction of the gelatinous layer towards the formation of the peduncles and possibly the outer layer of the spermatophore suggests a corresponding transformation in the chemical composition, especially proteins. The gelatinous layer and the peduncle stain intensely with Millon's reagent especially in fresh material suggesting the presence of tyrosine (Table IV). Dihydroxyphenols are not detected in any of the spermatophoric components using the ferric chloride test. However, strong phenolase activity is observed in the ventral gelatinous cord (Table IV). The crystalline spermatophore layer and the peduncle do not give a positive reaction to this test.

DISCUSSION

Pedunculate spermatophores are characteristic of Anomura. However, a macruran type of spermatophoric mass has been reported for a few members of the family Hippidae (Matthews, 1956; Subramoniam, 1977a). The present study suggests interesting possibilities in the evolution of pedunculate spermatophores from the non-pedunculate spermatophoric ribbon.

In both *A. symnista* and *E. asiatica* the spermatophore proper is supported by a basal gelatinous cord and an impregnating gelatinous matrix (Table V). This resembles several of the panulirid spiny lobster spermatophores, described by Berry and Heydorn (1970). In *A. symnista* the spermatophore is tubular with apparent node-like constrictions leading to internal discontinuities; in *E. asiatica* it is broken up into ampullae with drawn out peduncles. Interestingly, in two hermit crabs, *Dardanus asper* and *Pagurus novae zealandiae,* Matthews (1953) and Greenwood (1972) respectively, found evidence that the segmentation of the continuous sperm sheath leading to stalked spermatophore results from specialized muscular activity and modified lumen shape of the VD. The breaking up of the continuous spermatophoric tube by constrictions (*Albunea*) and distinct spermatophoric ampullae with drawn out peduncles set on a basal filamentous pedestal (*Emerita*) suggests that these anomuran sand crabs may be midway forms in the evolution of discrete pedunculate spermatophores of other anomurans from the tubular spermatophores of Macrura.

In the brachyuran decapods, the PVD is secretory and DVD has a storage function (Hinsch and Walker, 1974). However, the macruran and anomuran DVD too is highly secretory producing the accessory mucoid spermatophoric substances (Matthews, 1951; Berry and Heydorn, 1970). Furthermore, to increase the surface area of the glandular epithelial cells the DVD possesses typhlosole-like inpushings. In *E. asiatica,* the typhlosole corresponds to that of the spiny lobster, *Panulirus homarus* (Berry and Heydorn, 1970) in that the typhlosole consists of long columnar cells with mul-



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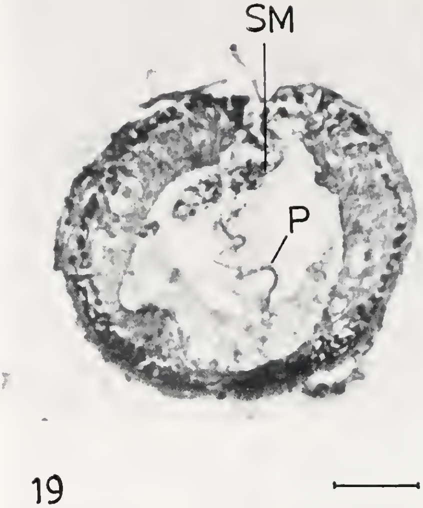

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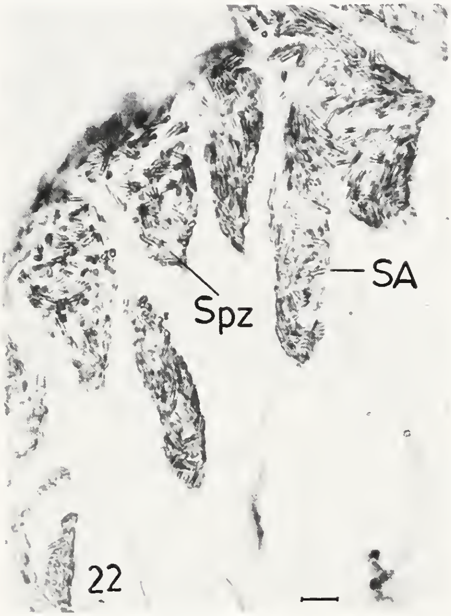

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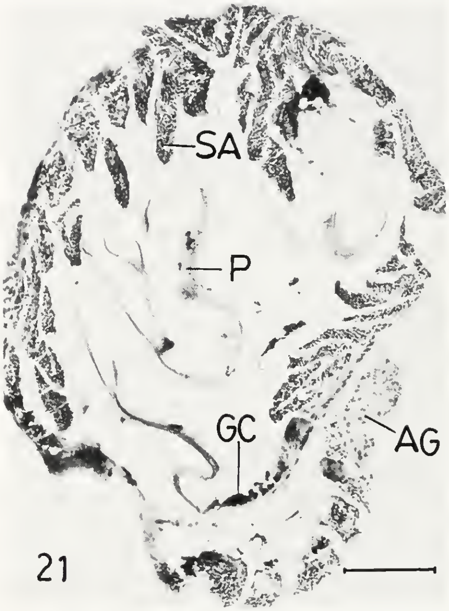

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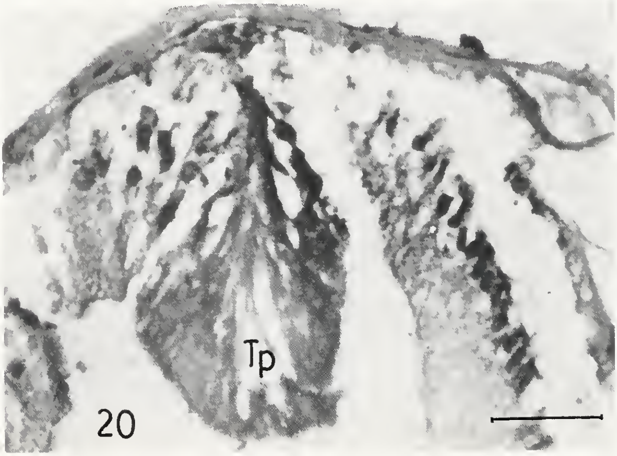

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tilobation at the peripheral region forming the shape of a leaf. However, in *A. symnista* there are two conical shaped dorsolaterally placed typhlosoles.

Mucopolysaccharides form the main components of the spermatophores of *A. symnista* and *E. asiatica.* AB-PAS differentiates the acidic mucopolysaccharides of the sperm mass-substance and the gelatinous matrix from the neutral mucopolysaccharides of the ventral gelatinous cord (Table V). Toluidine blue staining at different pHs indicates that the acidic mucopolysaccharides of the sperm mass substance and gelatinous matrix of *A. symnista* are rich in sulphated and carboxylated groups, respectively. Spicer (1960) suggests that mild methylation hydrolyses the sulphated groups and esterifies the carboxylated groups. With this treatment before the AB-PAS test, neither the —COOH groups of gelatinous matrix nor the $-SO_4$ groups in the mucopolysaccharides of sperm mass substance could be removed. However, prolonged methylation resulted in the removal of basophilia in sulphated acid-mucopolysaccharides and the assumption of PAS positivity suggesting the complete hydrolyses of $-SO_4$ groups. At the same time, the gelatinous matrix did not lose basophilia suggesting that the carboxylated groups may be enormous. These acidic groups may bind inorganic ions such as calcium ions (Aminoff *et al.,* 1970) from sea water to produce 'hardening' of the spermatophore into a putty-like mass.

The gelatinous matrix of *E. asiatica* contains periodate reactive acid mucopolysaccharides. Such a mucopolysaccharide heterogeneity has also been reported in the epithelial mucin of several vertebrate systems (Spicer and Duvenei, 1964) and in the vas deferens epithelial mucin of a pulmonate slug (Els, 1974). The predominance of various muco-substances in the spermatophoric components of the two sand crabs (see Table V) may be correlated to their protective as well as structural functions (Jeanloz, 1970; Montgomery, 1970). Though carbohydrate forms the major component of mucopolysaccharide substances, only meager amounts of glycogen are found suggesting that the spermatophoric components may not have a nutritive function. Interestingly, the sperm cells contain a significant quantity of glycogen, possibly for endogenous energy metabolism.

A similarity of the crustacean spermatophore to arthropod cuticle has been proposed because of the possible occurrence of phenolic tanning in the spermatophore wall (Malek and Bawab, 1971) and a chitin-protein like lamellar pattern (Gharagozlov-van Ginneken, 1978). In the spermatophores of *E. asiatica* (and to a lesser extent *A. symnista*), there is evidence for tyrosine-rich protein and the enzyme phenolase. However, no diphenols have been detected. Possibly, the tyrosyl residues, in the presence of phenolase might be oxidised *in situ* to quinones which tan the protein. This mechanism, called 'self-tanning' (Malek and Bawab, 1971; Hackman, 1974) has

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FIGURE 18. Transverse section through the anterior part of the PVD showing the columnar nature of the inner secretory epithelial cells (EC) and the agglutination of the spermatozoa (Spz) in the lumen. Bar = 20 μm.

FIGURE 19. Transverse section through another part of the PVD showing the sperm mass (SM) as well as the formation of the peduncle (P). Note the change in the shape of the lumen. Bar = 20 μm.

FIGURE 20. Transverse section through the proximal part of the DVD showing the leaf-like typhlosole (Tp). Note the absence of connective tissue inside the typhlosole. EC—epithelial cells. Bar = 50 μm.

FIGURE 21. Transverse section through the distal part of DVD showing the final arrangement of the spermatophoric ribbon with the spermatophoric ampule (SA) arranged on the dorso-lateral region. Each spermatophoric ampule is connected by the peduncle (P), originating from the ventral connecting cord (GC). AG—androgenic gland. Bar = 100 μm.

FIGURE 22. A closer view of the transverse section through spermatophoric ampule (SA) showing the haphazard arrangement of the rod-shaped spermatozoa (Spz). Bar = 10 μm.

TABLE III

Histochemical characteristics of mucopolysaccharide substances of spermatophoric mass of Emerita asiatica

Tests	Sperm mass*	Spermatophore Inner/Outer layer	Peduncle/ gelatinous cord	Gelatinous matrix
Best's carmine	+ R	+/+ R/R	++/++ R/R	+ R
Schiff alone	–	–/–	–/–	–
Periodic acid Schiff (PAS)	+++ M Sperm cells	+++/+ M/M	++/++ M/M	+ M
Alcian blue – PAS	++ M Sperm cells + B sperm mass substance	++/+ M/M	+/+ M/M	+++ B
Aldehyde fuchsin	++ B	+/– P/	–/–	+ P
Bracco-Curti	++ BB	–/–	–/–	–
Toluidine blue at different pH				
pH 1	++ V	+/– B/	±/±	+ V
pH 3	++ V	++/– B/	+/+ V/V	++ V
pH 4	++ BV	+++/– B/	+/+ V/V	+++ V
pH 7	++ B	+++/– V/	+/+ B/B	+ V
Alcian blue: critical electrolyte concentrations of $MgCl_2$				
0.2 *M*	+ B	+/± B/	+/+ B/B	++ B
0.6 *M*	+ B	+/– B/	–/–	++ B
0.8 *M*	±	±/±	–/–	++ B
1.0 *M*	+ B	–/–	–/–	++ B
1% Aqueous alcian blue	+ B	+/± B/	±/±	++ B
Chitosan	–	–/–	–/–	–

B = Blue; BB = Benzidine Blue; Br = Brown; BV = Bluish Violet; M = Magenta; P = Pink; R = Red; V = Violet; * = Sperm mass refers collectively to sperm mass substance, that binds the sperm cells within the spermatophore as well as the sperm cells. When reactions are distinct for sperm cells and sperm mass substance they are indicated accordingly.

TABLE IV

Histochemical characteristics of proteinaceous substances of spermatophoric mass of Emerita asiatica

Tests	Sperm mass	Spermatophore Inner/Outer layer	Peduncle/ gelatinous cord	Gelatinous matrix
0.1% Aqueous bromophenol blue	++ B	++/+ B/B	+/+ B/B	+ B
0.1% Aqueous toluidine blue (TB)	++ V	++/++ V/V	++/++ V/V	++ V
Dimethylaminobenzaldehyde (DMAB)	+ B	+/+ B/B	+/+ B/B	+ B
Millon's	−	−/−	+/+ BR/BR	+ BR
Performic acid alcian blue (PFAB)	−	−/−	−/−	++ B
Performic acid Schiff	+ P	+/− P/	−/−	−
Ferric ferricyanide	+	+/− PB/	−/−	−
Ferric chloride	−	−/−	−/−	−
Catechol incubation	−	−/−	+/+ Br/Br	−
Catechol + Pretreatment with formalin	−	−/−	++/++ Br/Br	−
Catechol + Pretreatment with alcohol	−	−/−	−/−	−
LIPID				
Sudan Black B	−	−/−	−/−	+ Bl.B
1% Nile blue	+ B	+/− B/	−/−	−

B = Blue; BR = Brick Red; Br = Brown; P = Pink; V = Violet; Bl.B = Bluish black.
− = negative; ± = doubtful; + = moderately positive; ++ = positive; +++ = intensely positive.

been reported for the adhesive cement of the cirripede, *Balanus crenatus* (Linder and Dooley, 1973). More importantly, the phenolic compounds may have other roles such as antimicrobial activity (Brunet, 1980) for the exposed spermatophores.

The structural difference in the spermatophores of *E. asiatica* and *A. symnista* may also be correlated with the mechanism of dehiscence. In *E. asiatica* the spermatophore deposition always occurs before egg extrusion and no dehiscence takes place until the egg mass comes in contact with the spermatophores (Subramoniam, 1977a; his Figs. 1G–1J). He also suggested that an oviductal secretion is responsible for the digestion of the cementing material closing the lip of the spermatophore. However, in *Albunea* this mechanism may not apply because the thick protective

Table V

Summary of the results concerning the characterization of mucopolysaccharides of the spermatophoric components of A. symnista *and* E. asiatica

Spermatophoric components	Chemical nature	Origin
Albunea symnista		
1. Sperm mass substance	Sulphated AMP	PVD
2. Spermatophore wall	Neutral MP	PVD
3. Gelatinous cord	Neutral MP	DVD, Ventral epithelium
4. Gelatinous matrix	Carboxylated AMP	DVD, Dorsal epithelium especially typhlosole.
Emerita asiatica		
1. Sperm mass substance	Sulphated AMP	PVD
2. Spermatophore wall	Carboxylated AMP	PVD
Inner layer		
Outer layer	Neutral MP	DVD, Ventral epithelium
3. Peduncle/gelatinous cord	Neutral MP	DVD, Ventral epithelium
4. Gelatinous matrix	Periodate reactive AMP	DVD, Dorsal epithelium especially typhlosole

matrix hardens into a putty-like substance on exposure to sea water. It is possible that the females use their powerful chelae to remove the protective matrix and then gouge the spermatophoric tube open to release the spermatozoa at the time of fertilization, as reported in the spiny lobsters (Fielder, 1964; Berry, 1969).

The two sand crabs, *E. asiatica* and *A. symnista* inhabit the shifting sands of the surf zone. Hence, the sticky spermatophoric ribbon in these crabs has great adaptive value because it can be deposited quickly and firmly. Further, in *E. asiatica,* spawning rapidly follows spermatophore deposition (Subramoniam, 1977b) and hence the spermatophoric ribbon remains as a jelly. Conversely, there seems to be a long time period between mating and spawning in *A. symnista* (unpub. obs.) with a necessity for the spermatophore to undergo 'hardening.' These factors may contribute to the reproductive success of the sand crabs, especially *E. asiatica* (Subramoniam, 1977b, 1979, 1981).

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DIETARY IMMUNOASSAY OF *ILYANASSA OBSOLETA*, THE EASTERN MUD SNAIL*

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ABSTRACT

The gut contents of mud snails from a high intertidal saltmarsh in South Carolina were examined both visually and immunologically from eight sample dates. Antisera to a variety of potential prey types were used in double-immunodiffusion tests of the amorphous gut material. Near absence of meiofaunal prey and presence of scant animal remains confirms the facultative carnivorous feeding mode for this species, while presence of frustules, sediments, and detritus indicated the dominance of herbivory and detritivory. *Ilyanassa obsoleta* is probably relatively unimportant as a predator on living benthic invertebrates, but it may be very important in detrital remineralization and physical breakdown processes.

INTRODUCTION

Feeding studies of the eastern mud snail, *Ilyanassa obsoleta* (Say), have provided nearly as many characterizations of its feeding mode as the number of studies conducted. It has been called primarily a deposit feeder (Pace *et al.,* 1979), an omnivorous deposit feeder (Nichols and Robertson, 1979), a facultative carrion-feeder (Gurin and Carr, 1971), a non-selective biological 'vacuum cleaner' (Curtis and Hurd, 1981), or a facultative herbivore/carnivore rather than an omnivore (Brown, 1969). Sheltema (1964), however, regarded the snail as principally herbivorous. Conner and Edgar (1982) found that living diatoms comprised a major portion of the snails' diet and surmised that dead foods were unimportant. It is remarkable that visual analysis of this common mollusc's gut contents could generate so many descriptions of its trophic mode. Much of the variability may stem from habitat heterogeneity or time of collection (see Robertson, 1979), but more likely than not, much of the classification difficulty derives from our inability to identify gut contents with traditional visual techniques.

Studies such as the above are typical for the gut contents analysis of marine benthic deposit feeders in that even qualitative estimates of the relative importance of different foods are made with little assurance of their accuracy. Direct observations of ingestion are nearly impossible with deposit-feeders, so we have had to rely on visual analysis of gut contents for dietary information. Despite the drawbacks of microscopical identifications, most of our knowledge of trophic interactions is derived from such studies. As part of a larger study of trophic connections among salt marsh benthic invertebrates, the stomach contents of *I. obsoleta* were examined using the immunological methods of Feller *et al.* (1979) which were designed to identify the soluble proteins in gut contents that could not be identified using traditional visual methods. Although subject to many of the same biases as visual analysis (*e.g.*, dif-

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ferential rates of digestion for different prey types, ingestion not necessarily implying assimilation, etc.), the immunological technique allows positive identification of prey when visual techniques do not. Furthermore, negative results with the immunological method are also informative in that they allow one to state that various prey types were not present in a particular gut sample. With visual analysis alone, one cannot state explicitly what was absent in the sample. Absence in this sense is determined by the lower limit of sensitivity of the immunological method.

Ilyanassa obsoleta were sampled periodically during the year to provide gut material on which parallel visual and immunological assays could be made. It was hoped that the immunoassays would provide data to test the hypotheses that mud snails eat meiofauna (Pace *et al.*, 1979), that *Spartina* detritus is not utilized directly (Wetzel, 1977), and that carnivory is facultative rather than obligatory for this species.

MATERIALS AND METHODS

Mud snails were collected at random during low tide from the high intertidal saltmarsh near Oyster Landing at North Inlet, South Carolina, (33°20′N, 70°10′W) on eight occasions from April, 1980, to June, 1981. The marsh is a typical southeastern *Spartina alterniflora* Loisel habitat which is covered to a depth of approximately 0.3 m twice per tidal day. On each sampling occasion, approximately 30 snails were sampled and frozen in the field on dry ice. Lots of five snails in the size range 15–19 mm total shell length were pooled for analysis. Within each five-snail lot, individuals were dissected and their entire digestive tract removed with forceps. Each gut was examined as a smear on a glass microscope slide with the aid of a dissecting microscope at 500× magnification. The guts of these five snails were then pooled and solubilized in 0.1 ml TES-saline [5 m*M* N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, 30 m*M* NaOH, and 150 m*M* NaCl] at pH 7.3. Thus the same material was examined both visually and immunologically.

For immunoassay, 20 μl of solubilized gut material was placed centrally in a plastic template on agarose surrounded by small wells containing antisera to potential prey utilizing the micro-Ouchterlony technique (Ouchterlony, 1968). Precipitin lines which formed between the wells by diffusion of solubilized gut material and peripheral antisera were counted. Each gut preparation was assayed in duplicate with antisera to 19 different potential prey present in the study area. Cross-reactions and homologous self-reactions between solubilized whole-organism extracts of each potential prey item and the entire antibody array are shown in Table I. Antiserum preparation followed Feller *et al.* (1979). No attempt was made to assay the snail guts for the presence of large, rare organisms that occur in the high marsh becuse either no antiserum was available at the time or it was deemed too unlikely that mud snails preyed on them alive. These included blue crabs, (*Callinectes sapidus*), burrowing ghost shrimp of the genus *Callianassa,* wharf crabs (*Sesarma* spp.), and various fishes. The small volumes of gut material present in the snails also precluded any attempts to standardize the total soluble protein content of each batch prior to immunoassay.

The algorithm described by Feller *et al.* (1979) was used to confirm the presence/absence of specific prey. An example of how this algorithm operates is shown in Table II using a hypothetical community of just six species. Since antiserum to a given prey organism may also produce precipitin lines with proteins from the predator's gut lining and/or different prey in the gut, the algorithm was designed to mathematically eliminate all precipitin line formations which could have resulted from these cross-reactions. The conservative nature of this algorithm thus provides a minimum estimate of the true number of different prey taxa consumed by a predator. Additional details of the algorithm have been discussed by Feller *et al.* (1979 [p. 67]).

TABLE 1

Maximum number of precipitin lines observed in extract-antiserum immunodiffusion cross-reaction tests

	Whole-Organism extracts																			
Antisera to:	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
A	*15*	1	6	10		8	7	1	1	1	2	2	2	2	2	3			1	1
B		*7*	1	2										1			1			
C	3	2	*11*	10		4	4						2		1	1				
D	9	1	8	*14*		4	6		2	5	2	3			3	2		1	2	2
E	5	1	2	5	*7*	4	3	1	2		1	2	3	1	1			1	2	
F	4	1	2	5		*13*	5		1	2	1	1		1		2				
G	5	5	2	2		8	*8*	1		1		1		1	2				1	
H	1	2	2	1		1	1	*11*	7	2		4	4	2	2	2	2	2	1	
I	1	1		2	1	2	2	5	*12*	3	4	7	3	4	3	2	2	2	5	
J	2			1		2	3	3	3	*9*	6	3	4	6	3	3	2	2	3	1
K	1	2		3	1	2	2	6	4	8	*13*	8	7	9	5	6		3	8	
L	2	2	2	3		3	3	4	7	5	8	*15*	4	6	5	2		2	4	
M		2	4			2	5	3	5	3	6	7	*12*	5	6	5	2	3	3	2
N	1		2	3		2	3	3	2	4	5	7	7	*12*		3		1	6	
O		1	2	2			2	4	4	3	3	7	9	5	*14*	1		1	4	
P	1		2	1		2	2	2			2	2	2	2	3	*12*	2	2	3	
Q		1		1			3	1		1				1	1	1	*6*		1	1
R				1					1	1								*6*		
S		1	1	4		3	2	3	3	4	2	5	4	4	3	4		2	*11*	
T		1	1																	*8*

A = *Penaeus setiferus* (white shrimp)
B = Harpacticoid copepods
C = *Uca pugnax* (fiddler crab)
D = *Uca pugilator* (fiddler crab)
E = Ostracods
F = *Palaemonetes pugio* adults (grass shrimp)
G = *Palaemonetes pugio* juveniles (grass shrimp)
H = *Crassostrea virginica* spat (oyster)
I = *Crassostrea virginica* adult (oyster)
J = *Mercenaria mercenaria* spat (hard clam)
K = *Mercenaria mercenaria* adult (hard clam)
L = *Geukensia demissa* (mussel)
M = *Littorina irrorata* (periwinkle)
N = *Tagelus plebius* (razor clam)
O = *Ilyanassa obsoleta* (mud snail)
P = Oligochaetes
Q = Nematodes
R = Turbellaria
S = *Diopatra cuprea* (polychaete)
T = *Spartina alterniflora* (marsh cordgrass)

Italicized numbers on diagonal refer to self-reactions. Blanks denote no cross-reaction. These data are equivalent to Table II, part A.

TABLE II

Hypothetical example using the immunoassay algorithm

A)		Whole-organism extracts of:					
		I	II	III	IV	V	VI
Antisera to:	I	*8*		1	3	2	
	II	1	*5*		1	2	2
	III	2	3	*7*			1
	IV	2		1	*9*	6	1
	V		2		5	*10*	3
	VI	1	1	1		4	*13*

B)		# lines observed in gut of VI / # lines in self-reaction	Rank
Antisera to:	I	6/8 = 75.0%	1
	II	0/5 = 0	5
	III	3/7 = 42.9	3
	IV	5/9 = 55.6	2
	V	2/10 = 20.0	4
	VI[a]	13/13 = 100.0	—

C) Antiserum to: (by rank)	# lines observed in B above		# lines due to cross-reactions with extracts of:				
			VI		I		IV
I[d]	6	—	0	—	0[b]	—	3 = 3
IV[d]	5	—	1	—	2	—	0 = 2
III[c]	3	—	1	—	2	—	0 = 0
V[c]	2	—	3	—	0	—	5 = neg
II[c]	0	—	2	—	1	—	1 = neg

[a] Not ranked since VI is the predator.

[b] By definition no antiserum cross-reacts with itself.

[c] Eliminated by algorithm due to excessive cross-reaction.

[d] Confirmed as prey in gut of organism VI.

(A) Cross-reaction matrix showing maximum number of precipitin lines observed in double-immunodiffusion reactions between antisera to and whole-organism extracts of organisms I–VI; italicized numbers on diagonal refer to self-reactions, and blanks denote that no precipitin line was observed; (B) Results of immunodiffusion tests on the solubilized gut contents of organism VI (the predator); the number of lines observed with each antiserum is ranked by its proportion of the appropriate self-reaction, with the highest proportion ranked first; (C) Mathematical subtraction of lines which could have been due simply to cross-reactions with other prey organisms in the gut; if the number of lines observed with an antiserum minus the numbers due to cross-reactions with other potential prey is zero or negative, then that organism is eliminated from consideration as a prey.

RESULTS

Microscopic visual examination of the mud snail gut smears revealed only the following: amorphous brown mush, amorphous green mush, golden-brown mush, pieces of pennate diatom frustules, colorless fluids, sediment, sand grains, and "detritus." At no time were animal remains evident. All guts contained at least a little of each of the food items listed above, *i.e.*, no guts were empty. Sediments of silt and clay sizes comprised most of the volume of any individual gut, usually between 50 and 75%.

Immunoassays revealed several notable results. First, most of the precipitin line formations between gut contents and the various antisera (Table III) either were or could have been due to simple cross-reactions rather than actual presence of specific prey proteins. Second, there were no differences in gut contents between daytime low tide and nighttime low tide collections in August, 1980. Third, the absence of crustacean and living *Spartina* proteins in the guts indicates that these potential foods are not common in the mud snail diet. Fourth, meiofauna don't appear to be a major prey item, although harpacticoid copepod proteins were present in at least one snail's gut on 20 November 1981. Fifth, it is unknown whether the *Crassostrea virginica* proteins

TABLE III

Maximum number of precipitin lines observed in either of two replicate immunoassays of Ilyanassa obsoleta *guts*

Year	1980							1981		
Date	2 Apr	25 Aug		26 Aug		20 Nov	8 Dec	6 Jan	6 May	2 June
Time	1300	0130	1300	0200	1600	1230	1500	1430	1600	1400
Antisera to:										
A	1	1				1	1	1	1	
B	1					2*	1			
C										
D										
E										
F										
G										
H										1
I		1					1	6*	6*	4*
J	1	3	2	2	1	3			2	2
K	1	3	2	2	2	2	3		2	2
L	3	3	2	3	3	4	3	4	1	1
M	2	3	6	2	2	2	2	2	3	2
N	2			3			1	1		
O	14	14	14	14	14	14	14	14	14	14
P	2	2	2	2	1	2		1	2	3
Q										
R										
S							1	1		
T										

Two groups of five combined snail guts were tested each time. Blanks indicate that no lines were observed. Asterisk denotes presence of prey proteins as confirmed by algorithm of Table II. All other lines were probably due to cross-reactions as seen in Table I matrix. This data set is thus equivalent to that of Table II, part B. See Table I for antiserum identification.

present in 1981 were due to ingestion of living or dead oyster meat. Lastly, carnivory and/or carrion-feeding was not obligatory for mud snails in this size range.

DISCUSSION

These data are consistent with the hypothesis that *Ilyanassa obsoleta* is indeed a facultative carrion feeder and that herbivory and/or detritivory are the dominant feeding modes for this abundant, ecologically successful, marine deposit feeder. The visual analyses performed did not detect any semblance of carnivory, whereas the immunoassay did. Beyond this finding, however, many potential prey items that one might reasonably expect to have been present were not found in the diet of the mud snail. Chief among these were meiofaunal taxa, particularly nematodes, oligochaetes, and turbellarians. All of these taxa are abundant in the surface sediments of the high marsh where the mud snails were collected (Bell, 1979). Antisera to these meiofaunal groups can detect microgram quantities of protein, so it is unlikely that such prey would resist immunodetection if they had been present in the guts within 24 hours prior to when snail samples were collected and frozen. I take this as strong evidence that meiofauna are not a prominent dietary component of the mud snail, although Alexander (1979) found some meiofauna in the guts of periwinkle snails in a Louisiana salt marsh. Curtis and Hurd (1981) found nematodes in only a few mud snails.

Since the only *Spartina* antiserum available at the time of this study was prepared by injecting rabbits with a soluble protein extract (antigen) made from standing live green plants, the absence of any precipitin reaction with the gut contents using this antiserum was not surprising. Ingestion of living plant tissue by the snails would probably have been detected had it occurred. The presence of amorphous colored mush and detritus was likely due in part to ingestion of aged, dead *Spartina* fragments. Antisera to extracts of aged *Spartina* detritus are presently in preparation. Hopefully these will successfully distinguish among true *Spartina* detrital proteins and proteins from bacteria and fungi attached to the detritus. It will then be possible to confirm actual ingestion of detrital proteins by the snails or any other detritivore and substantiate or refute the role of attached microflora in detritus-based food webs (*e.g.*, Newell, 1965; Adams and Angelovic, 1970).

The algorithm of Table II for confirming the presence of specific proteins is an indirect attempt to ensure that the precipitin lines observed are correctly ascribed to gut contents rather than cross-reactions. Direct evidence to confirm the presence of specific prey should be obtained by generating precipitin lines of identity between gut contents and a preparation of the actual prey protein using the antiserum specific to that particular prey. Although not done in this mud snail study, such tests in other trophic studies have established the reliability of the algorithm (pers. obs.). The algorithm does not, however, enable one to distinguish whether predators ate living or freshly dead tissue. The presence of *Crassostrea virginica* protein in snail guts in 1981 is more likely to have come from ingestion of dead oysters, as racoons frequently feed on oysters and leave debris in the high marsh. Young oysters are probably too large and thick-shelled for mud snails to penetrate. The harpacticoid copepods eaten on 20 November 1980 were also probably ingested dead, as most species present in the high marsh are mobile enough to escape the slower mud snail even though the copepods are several orders of magnitude smaller than the snails.

Robertson (1979) states that mud snails have crystalline styles and feed primarily when covered by the tide. Presence of a crystalline style, however, is not necessarily indicative of prior feeding (Curtis and Hurd, 1981). All snails had crystalline styles and were sampled within 30 min of their exposure to air at low tide. Each snail collected was also active on the sediment surface. Thus there was probably no bias introduced by inclusion in any of the samples of snails which had not recently fed.

The immunoassay as used in this study is a powerful qualitative tool for defining trophic pathways. It is limited only by the availability and sensitivity of antibodies specific to target organisms of interest. The method is not yet quantitative, however, so caution must be taken in the interpretation of its results. Only the presence, absence, or concentration of specific proteins in the predator's gut can be measured. Extrapolation of, for instance, a harpacticoid copepod protein concentration in a predator's gut to the number of copepods ingested by that predator is at present unwarranted. Rapid analysis of samples soon after collection is recommended, since proteolytic activity in the predatory snails' gut contents continues even during the 48 h incubation period of the immunodiffusion tests. Some loss of immunologically identifiable gut material may result from this proteolysis.

The mud snail's role in marine ecosystems may center on its mechanical processing of surface sediments and its associated detritus (see Conner *et al.*, 1982, and Edwards and Welsh, 1982) for it is not preyed upon by very many other organisms (Brenchley, 1982). Since Levinton and Stewart (1982) did not determine whether their mud snails actually ingested oligochaetes, the bioturbation effects of the mud snails may have been more important in the successional pattern observed than their direct biological effects. If the results of this study are indicative of its general dietary behavior in the intertidal ecosystem, then *I. obsoleta* is probably relatively unimportant as a predator on living marine invertebrates. However, as Curits and Hurd (1981) point out, by virtue of its high numerical abundance, *I. obsoleta* could still exert considerable influence on other members of the benthic community even if it consumes small numbers of prey. As suggested by Nichols and Robertson (1979) its greatest biological effect may be one of competition with other herbivores.

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THE EFFECT OF LOW DENSITY ON THE DEVELOPMENT OF SIMULTANEOUS HERMAPHRODITISM IN MALE *CAPITELLA* SPECIES I (POLYCHAETA)

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ABSTRACT

In this study it was shown that male *Capitella* species I may develop into simultaneous hermaphrodites if reared in isolation or at low densities in the laboratory. Since hermaphroditic development is rare in high density non-inbred cultures but becomes frequent in inbred lines which have been selected for high male sex ratios, the ability to become hermaphroditic seems to have a genetic basis. It is also suggested that low density may trigger the hermaphroditic development of males through the absence of animals with female gonads. Excess food resources which could be assimilated at low density and may be directed into the development of a second set of reproductive structures and female gametes, could also be a factor in the male-to-hermaphrodite switch. *Capitella* sp. I has an opportunistic lifestyle which could accommodate such a sexual adaption. The larvae are widely dispersed which would often cause adults to be at low density where the advantage of being hermaphroditic is well known.

INTRODUCTION

Much of the scientific interest in the genus *Capitella* stems from its "opportunistic" life history (Grassle and Grassle, 1974) which has made it an important indicator of environmental disturbance in the benthos (Henrikkson, 1969; Pearson and Rosenberg, 1978; Sanders *et al.,* 1980). Formerly known as *Capitella capitata,* electrophoresis has shown it to be a complex of at least ten sibling species (Grassle and Grassle, 1976, 1977; Grassle, 1980). The different species are morphologically similar but differ in life histories and reproductive strategies. The most opportunistic species, which is distinguished by larvae which remain in the planktonic stage for several hours, is *Capitella* species I. This species is the main one under consideration here.

Males, females, and hermaphrodites are found in natural populations or *Capitella* sp. I. Hermaphrodites are found only infrequently, appearing when mature males develop female gonads. Families raised in the laboratory from individual eggcases have extremely variable sex ratios and successful selection can be made for inbred lines with a high proportion of males and females (Grassle, 1980). Both facts suggest that there may be a polygenic sex determining mechanism (Kossig, 1964). If selection is made for a highly male sex ratio, the proportion of hermaphrodites that develop will also increase. The discovery that motivated this research was that young non-inbred males of species I and II developed female gonads, thus becoming hermaphrodites, if reared in isolation. Previously, hermaphrodites were observed to develop in old, high density, non-inbred laboratory cultures only. Hermaphrodites are never

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found in *Capitella* species IIIa and all individuals of species III are hermaphroditic (Grassle, 1980).

This developmental response to isolation leads to several questions concerning the proximate stimuli causing hermaphroditism and its ultimate role in the overall reproductive strategy of the animal. The first question examined here was whether these young hermaphrodites function simultaneously as males and females. Secondly, the effects of density on hermaphroditic development were investigated. This is particularly interesting when the adaptive significance of the mixture of reproductive modes is considered. Low density is frequently cited as an important factor in the evolution of hermaphroditism (Tomlinson, 1966; Ghiselin, 1969; Maynard Smith, 1978; Borgia and Blick, 1981). Since *Capitella* may have a polygenic sex determining mechanism, it is also of interest to see how potential differences in degree of maleness and femaleness, caused by differences in sex determining alleles, may affect the rate of development of hermaphroditism in isolated males.

MATERIALS AND METHODS

Two stations in New Bedford Harbor, Massachusetts have been sampled at regular intervals with 1/25 m^2 Van Veen grab from November 1981 to the present. The first, Sewer West in the outer harbor, has had low densities of *Capitella* sp. I (<0.1 worms/cm^2) and occasionally a few individuals of *Capitella* sp. III, on each sampling occasion. Station 3 in the inner harbor has revealed densities of *Capitella* spp. I and II of 4 to 6 worms/cm^2. Two samples from Sewer West and one sample from Station 3 containing hermaphrodites were sexed and subsequently sacrificed and identified to species by electrophoresis.

Males of several sibling species of *Capitella* were isolated from a field collection from the Woods Hole Sewer Outfall (6-1-81), and from a laboratory aquarium (13-1-81) with a population of *Capitella* derived from larvae entering the aquarium in the unfiltered sea water lines. Individual males were kept in filtered sea water at 15°C, fed to excess, and examined for macroscopic evidence of oögenesis at approximately weekly intervals up to 34 days. Survivors were identified to species by electrophoresis.

All of the animals used in the following experiments were *Capitella* species I and originated from laboratory stocks at the Marine Biological Laboratory, Woods Hole, Massachusetts in 1980. They were cultured at 20°C in plastic or glass containers of various sizes in a medium of mud and sea water. The mud, which supplies the food source for these animals, was collected from several intertidal sites on the eastern shore of Nova Scotia. To prepare the mud it is sieved through screens of standard size 20 (841 μ) and then frozen until use. The mud and sea water were replaced at weekly intervals in all experiments. No attempt was made to standardize the nutrients available to each animal. Food was always added in excess.

(1) To check whether hermaphrodites are functional as males and females simultaneously, 16 40-day-old males from a non-inbred line were isolated. All had become fully developed hermaphrodites after 25 days of isolation; at that time 7 were paired with 7 known males and 7 more were paired with 7 known females. The females had not been in contact with any males for 2 weeks prior to the experiment so there was a low possibility of sperm storage. Pairs were checked for eggcases at weekly intervals.

(2) Sibling males from many different families were used to determine the effect of density on hermaphroditic development. Here the term family will be used to mean a group of animals that are siblings. Families of known age were reared separately

at different densities from 0.02 worms/cm^2 to 0.19 worms/cm^2. Worms/cm^2 is used as a measure of density since *Capitella* sp. I generally feeds near the surface. Each of the family groups were sexed at weekly intervals for a period of 6 weeks. Hermaphrodites were not removed from the cultures as they developed. The influence of age of the families and their densities on the resulting proportions of hermaphrodites were analyzed by multiple regression.

(3) Variations in the time taken by isolated males to become hermaphrodites were studied using 112 males from 6 non-related families. All animals were 66–78 days-old. Fifty-six males came from 3 families which had more females than males. At the time of sexing the ratios of these 3 families were 4 ♂:7 ♀:1 juvenile, 60 ♂:69 ♀:1 juvenile and 32 ♂:41 ♀:19 juveniles. The original number of larvae set out in these families were 30, 200, and 100 respectively. The rest of the males came from families with a very high proportion of males and were, therefore, perhaps different from the others in their sex determining genes. The sex ratios of this second group of families were 65 ♂:2 ♀:8 juveniles, 20 ♂:2 ♀:9 juveniles and 76 ♂:8 ♀:8 juveniles. The original number of larvae set out in these families were 100, 50, and 100 respectively. All the males were isolated under identical conditions and checked for the development of female gonads every 2 days for 38 days. At that time the number of hermaphrodites was constant. Later, several of the males which switched to hermaphrodites early, after less than 9 days of isolation, were cultured together and a mixed batch of the resulting larvae were allowed to develop. At 69 days of age, 32 males from this group of offspring were isolated in the same manner as their parents and observed for 20 days.

RESULTS

Hermaphrodites are found at very low densities in the field. In all the *Capitella* individuals examined at capture from Sewer West and Station 3 field stations, and that were subsequently identified electrophoretically as *Capitella* sp. I or II, only four hermaphrodites belonging to *Capitella* sp. I, were found (Table I). Table II shows that both *Capitella* sp. I and II may develop into hermaphrodites when isolated.

In the experiment to check for the functionality of simultaneous hermaphroditism, 5 of the 7 females paired with hermaphrodites were found in fertile eggcases within 14 days after the cultures were set up. There had also been successful fertilization of all hermaphrodites that had been cultured with males, demonstrating that hermaphrodites that are induced to develop through isolation can function as simultaneous hermaphrodites.

Figure 1 shows the relationship between density and the proportion of males turning to hermaphrodites in the all-male mass cultures of varying density. The multiple regression performed on the results shows that both density and age have

TABLE I

Number of Capitella *sp. I hermaphrodites found in 3 field samples of varying density*

Station/Sampling date	Density Worms/cm^2 *Capitella* spp.	Number of *Capitella* sp. 1 hermaphrodites	Total *Capitella* spp. individuals
Sewer West/21-12-81	0.015	2	6
Sewer West/28-6-82	0.045	1	18
Station 3/2-12-82	4.079	1	302

TABLE II

Number of Capitella *spp. I, II, and IIIa males switching to hermaphrodites and the time in which they switched under isolated conditions*

Source population	Number of *Capitella* males isolated	Number of *Capitella* dying in 34 days without switching	Number of *Capitella* of spp. I, II, and IIIa	Number switching to ⚥ in 34 days or less	Mean days to switch	Range
Woods Hole	26	6	2 sp. I	2	—	14–27
Sewer			17 sp. II	15	25.7	13–34
Outfall			1 sp. IIIa	0	—	—
Aquarium	8	1	7 sp. I	7	22.0	13–27

a significant effect on the proportion of males turned to hermaphrodites (ANOVA F-ratio = 15.06, $P < .05$). The interaction between these two factors is not significant. The lower the density and the greater the age of the animals at the beginning of the experiment, the higher the proportion of hermaphrodites that develop.

Ninety-six of the 112 males used in the isolation experiment lived through the 38 day period of observation, 54 from families with more females than males and 42 from families with a very high male sex ratio. The peak period of male to hermaphrodite sex change was between 7 and 15 days after isolation, during which time 76% of the males developed female gonads.

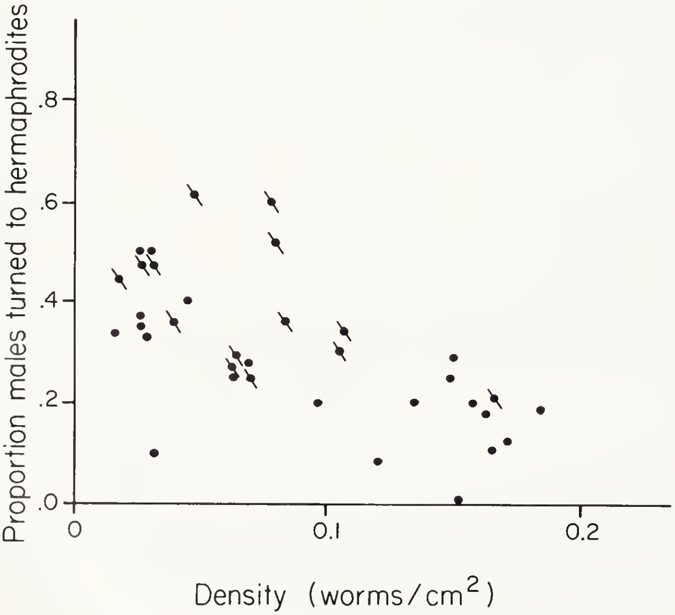

FIGURE 1. Proportion of males turned to hermaphrodites when all-male groups are cultured at varying densities. Each point represents an all-male, non-inbred family group of variable age. ● represents families which were over 70 days of age when the experiment commenced.

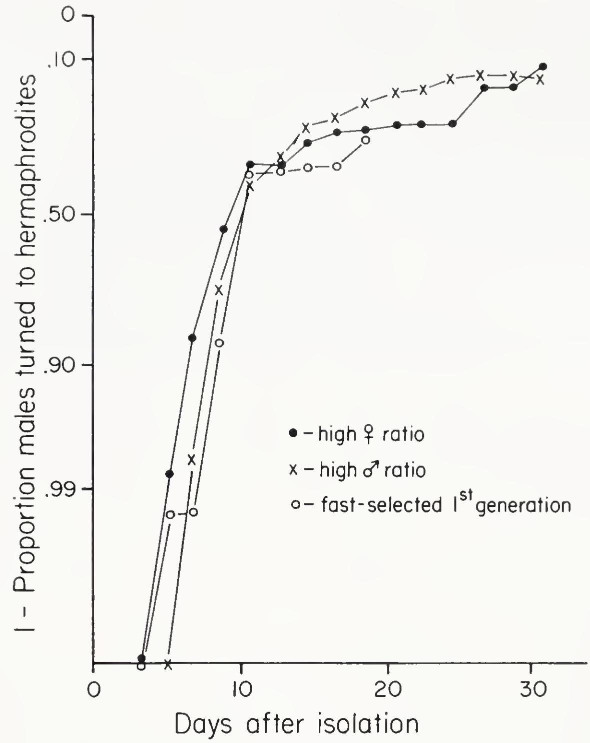

FIGURE 2. Probability plots of proportion of males turned to hermaphrodites in the high male ratio group, high female ratio group and fast-selected first generation against time in days.

Figure 2 shows that there is little observable difference in the proportion of males developing into hermaphrodites between the 2 types of families (high and low male) used as parents. Tests of proportions (Sokal and Rohlf, 1969) between the different sex ratio groups were not significantly different ($P > .05$) for 14 out of 16 2-day intervals during which change was occurring. Twenty-seven out of 32 animals survived the 20-day period of observation in the first selected generation. Tests on the proportion of males switching to hermaphrodites over the 2-day intervals through 20 days show that 4 of the 9 intervals tested are significantly different between the selected generation and the pooled parent generation. Three of the 4 intervals were during the peak period of change from 7–15 days. Surprisingly, the fast-selected line switched later than the pooled parental lines.

It was observed that more food, in the form of the particulate matter (not compacted into fecal pellets) was present at the end of the weekly food replacement cycle in the low density and isolated cultures.

DISCUSSION

Low density promotes the development of female gonads in male *Capitella* sp. I resulting in simultaneously hermaphroditic animals. How this aspect of the environment (low population density) produces such an effect may be explained in two ways. Low density and isolation may be promoting the sex change through increased food availability or the phenomenon may be induced through the absence of inhibitory effects from animals in close proximity which have female gonads.

The individually isolated animals had a constant and abundant food supply and in the density experiments, the low density cultures had a high amount of food available per animal. In both instances there would be an excess of nutrients available and extra energy might be channeled into the development of the second, female set of reproductive structures and gametes without detracting from any part of the normal energy budget of an animal. This is assuming that the budget is not time-limited. Also, in both experiments only males were used and the results could have been affected by the absence of animals with female gonads. It remains to be seen whether a lack of hormonal secretions in the environment, such as pheromones produced by the females, will trigger the development of female gonads in males. The mechanism by which the age of an animal may affect its ability to become hermaphroditic is also unknown.

The results of the isolation experiment appear to show that possible differences in sex determining genotype do not play a role in the response of males to isolation. If differences in sex-determining genes actually exist in the animals used, they do not affect the rapidity of the male sex change in isolation. However, the ability to actually change sex is genetically controlled and can be selected for, as shown by the fact that inbreeding causes selection for the ability to undergo sex change. The fact that some animals never changed sex in the isolation experiment implies that there is an underlying genetic basis to the expression of the phenomenon.

Since *Capitella* sp. I is a widely dispersed opportunistic species, animals occasionally find themselves at very low densities where finding a mate of the opposite sex would be unlikely. The genes controlling the ability to change sex may, therefore, be selected for in *Capitella* as an 'emergency adaption.' The development of two sets of reproductive structures, although potentially energetically expensive, would increase the chances of finding a suitable mate. The ability to self-fertilize would, of course, be advantageous as well, but there is little evidence that *Capitella* sp. I self-fertilize and the pronounced inbreeding effects in this species are further evidence that selfing is uncommon. In conclusion, it seems that the establishment or maintenance of hermaphroditism as a reproductive strategy may be selected for because of the opportunistic tendencies of this animal.

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SYMBIOSIS BETWEEN THE ZOOXANTHELLA *SYMBIODINIUM* (= *GYMNODINIUM*) *MICROADRIATICUM* (FREUDENTHAL) AND FOUR SPECIES OF NUDIBRANCHS

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ABSTRACT

The dinoflagellate *Symbiodinium* (= *Gymnodinum*) *microadriaticum* (Freudenthal) occurs in a symbiotic association with the nudibranchs *Melibe pilosa,* an undescribed *Melibe* sp., *Pteraeolidea ianthina,* and *Berghia major.* The algal symbionts reside in host-derived "carrier" cells associated with the host's digestive gland. Longer survival of starved *M. pilosa, P. ianthina,* and *B. major* in constant light than in constant dark indicates that photosynthetically fixed carbon is translocated from symbiont to host. Large lipid deposits, present in the same animal cells that contain zooxanthellae in *Melibe* sp. and *P. ianthina,* suggest that lipid or lipid precursors may comprise part of the translocated nutrients in these species. A large proportion of the fecal material in *Melibe* sp., *P. ianthina,* and *B. major* is composed of degenerate algal cells. It is possible that these species obtain part or all of their translocated nutrients by digestion of some of their algal symbionts. An organ that appears to function as the site of zooxanthella digestion is present in the cerata of *P. ianthina.* Zygotes and larvae of all four nudibranch species are devoid of symbionts, thus each new host generation must be re-infected with *S. microadriaticum.* Adults of *B. major* feed on prey that contain symbiotic zooxanthellae while the other three nudibranch species examined do not. These facts suggest that while the historic inception of the symbiosis in *B. major* was probably secondary, the symbionts being derived from the prey; in *M. pilosa, Melibe* sp., and *P. ianthina* the inception may have been primary, the symbionts being obtained by inadvertant ingestion by the host.

INTRODUCTION

The symbiotic relationships between zooxanthellae and a number of different invertebrate hosts have been of biological interest since the late 1800's (*e.g.,* Brandt, 1881), initially as a zoological curiosity and more recently as a means of studying the interactions between genomes of different evolutionary origins that are in intimate cytoplasmic contact. While a considerable amount of work has been done on tridacnid clams (Kawaguti, 1966; Muscatine, 1967; Fankboner, 1971; Goreau *et al.,* 1973; Fitt and Trench, 1981; Trench *et al.,* 1981) and coelenterates (Boschma, 1925; Muscatine and Hand, 1958; Muscatine, 1967; Taylor, 1969a, b; Trench, 1971a, b, c; 1974; and many others), little consideration has been given to associations between zooxanthellae and various nudibranch species. Inquiries that have investigated this symbiosis have been descriptive in nature and, aside from the recent work of Rudman (1979, 1981a, b, 1982), have lacked detail and comparative analysis (Hornell, 1909; Naville, 1926; Rousseau, 1931). Research concerned with the physiology of nudibranch-zooxanthellae associations is totally lacking in the literature.

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The fact that different species of these complex organisms have varying degrees of morphological modification to accomodate the symbiont (Rudman, 1981a, b; 1982) suggests that host-symbiont interaction is not the same in all nudibranch species. Analysis of the physiological aspects of these associations should allow further insight into the evolution, inception, maintenance, and control of algal-invertebrate symbioses.

At least seven species of nudibranchs found in Hawaiian waters maintain a symbiosis with zooxanthellae. These are the dendronotids *Melibe pilosa,* and two undescribed *Melibe* species, and the aeolids *Berghia major* (possibly the same as *Spirulla major* as re-described by Rudman, 1981b), *Pteraeolidea ianthina, Baeolidea nodosa,* and one unidentified aeolid species. The following research considers the nature of the symbiotic association in *M. pilosa,* one of the undescribed *Melibe* species (hereafter termed *Melibe* sp.), *B. major,* and *P. ianthina.*

MATERIALS AND METHODS

Collection and maintenance of animals and egg masses

Animals were collected in the field by skin and SCUBA diving and were held in the lab under constant light in beakers or glass finger bowls containing pre-filtered (Millipore Cat. no. AP2504700) sea water (hereafter termed PFSW). Water in these containers was changed daily in most instances. Taxonomic identifications of the described species are based on descriptions given by Kay (1979). Egg masses laid in the lab were incubated through hatching in beakers of aerated PFSW that were changed daily.

Identification of host prey

Prey of the nudibranchs examined in this study were determined from examinations of the fecal material of recently collected animals and/or observations in the lab of feeding on prey items found in each species' habitat. In *Pteraeolidea ianthina,* the nematocysts stored in the cnidosacs of the cerata were also examined and compared to those of various coelenterates found in its habitat.

Algal taxonomy

Taxonomic identification of the zooxanthella associated with the nudibranchs was made by comparison of the alga's morphological characteristics, as seen with the light and electron microscopes, with the descriptions of Freudenthal (1962) and Kevin *et al.* (1969). Zooxanthellae were separated from host tissues by homogenizing specimens in a glass tissue grinder with plastic plunger and then washing by centrifugation as described by Muscatine (1967). The algae were then placed in either sea water or Provasoli's E.S. medium (Provasoli, 1968) in constant dark overnight. Motile stages were examind with a compound microscope the next day after the separated algal cells had been exposed to a few hours of light. Motile stages were also produced by a similar incubation of either host fecal pellets or rotting host tissues in sea water.

Translocation of nutrients from symbiont to host

Starvation experiments were performed in constant light or constant dark to test the hypothesis that nutrients, in the form of photosynthetically fixed carbon, are translocated from the zooxanthellae to the host. Similar-sized pairs of animals were starved in containers filled with 0.45 μm Millipore filtered sea water (hereafter termed MFSW) that contained the antibiotics streptomycin sulfate and penicillin G at concentrations of 50 and 60 μg/ml respectively (Switzer-Dunlap and Hadfield, 1977) with (*Melibe pilosa* and *Berghia major*) or without (*Pteraeolidea ianthina*) aeration. Animal containers and medium were changed daily. The wet mass of the starved animals was determined on a regular basis; however, loss of cerata in *M. pilosa* obscured changes in mass for this animal.

Host defecation of healthy and degenerate zooxanthellae

Squashes of fecal material from *Melibe pilosa, Melibe* sp., *Berghia major,* and *Pteraeolidea ianthina* were prepared and the healthy and degenerate algal cells in 10 equal areas from each squash were counted under a compound microscope. Results were converted to percentages of the total number of cells in each area. Cells were considered healthy if they were spherical with a smooth border and contained an identifiable chloroplast and other organelles. Degenerate cells were those that had an irregular cell boarder, were shrunken in appearance, and lacked distinct organellar organization. The significance of the change in the results for fecal counts of *Melibe* sp. that were either a) unstarved or starved for 1 day or b) starved for 3 days was determined by comparing the differences between mean percent healthy and mean percent degenerate algal cells in each animal of the two ranked groups with a Mann-Whitney U test (Seigel, 1956).

Identification of lipid in host tissues

Larvae and small, whole cerata of *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* were relaxed in a mixture of three parts PFSW and one part MFSW saturated with chlorobutanol and then fixed in 10% formalin in sea water. Fixed material was dehydrated through an ethylene glycol:distilled water series (1:3, 1:1, 3:1, 100% ethylene glycol; 5 min/treatment) and then transferred to a saturated solution of Sudan IV in ethylene glycol (Chiffelle and Putt, 1951) for 3 to 8 h (modification of method of Williams-Arnold, 1974). Stained material was rinsed in 100% ethylene glycol to remove excess stain and then examined with a compound microscope.

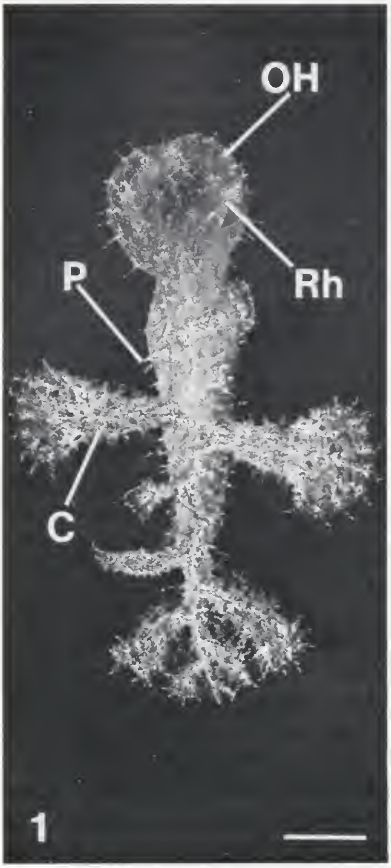
Light and electron microscopy

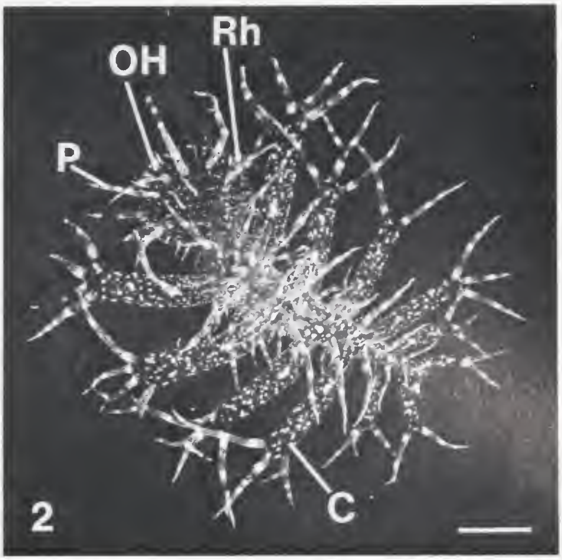
Tissues were relaxed in the chlorobutanol:sea water solution described above and then fixed in 2.5% Millonig's phosphate buffered glutaraldehyde (Cloney and Florey, 1968) for one h. The tissues were then washed in either 2.5% $NaHCO_3$ or a 1:1 mixture of 0.6 *M* NaCl and Millonig's phosphate buffer and post-fixed in 2% OsO_4 in 1.25% $NaHCO_3$ for one h (Wood and Luft, 1965). Fecal pellets of *Melibe* sp. were fixed using the same procedure, but were not placed in the chlorobutanol:sea water mixture. Dehydration was carried out in an ethanol series through 100% propylene oxide and the tissues were embedded in EPON (Luft, 1961). Sections for light microscopy were stained with Richardson's stain (Richardson *et al.,* 1960). Ultrathin sections were stained with uranyl acetate and/or lead citrate.

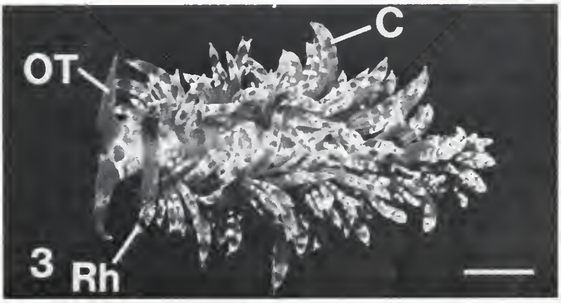
RESULTS

Host prey

Adults of both *M. pilosa* (Fig. 1) and *Melibe* sp. (Fig. 2) feed on small crustaceans (*i.e.,* copepods, isopods, etc.) that are captured with their expandable oral hood. The exoskeletal remains of these prey are quite evident in the fecal material of freshly collected specimens of both *Melibe* species and their feeding on newly hatched brine







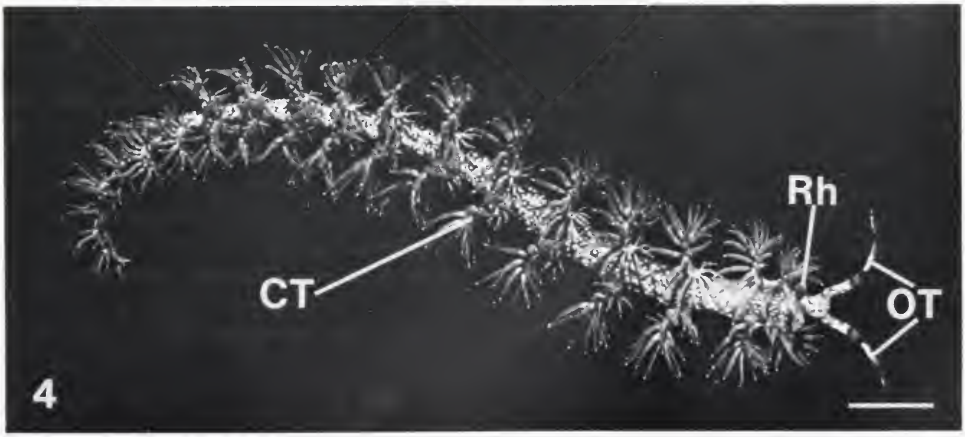


FIGURE 1. Adult *Melibe pilosa* (at least 3 cerata are missing). C–ceras, OH–oral hood, P–papilla, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 2. Unidentified, adult, *Melibe* species. C–ceras, OH–oral hood, P–papilla, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 3. Adult *Berghia major*. C–ceras, OT–oral tentacle, Rh–rhinophore. Bar ~ 1.0 cm.

FIGURE 4. Adult *Pteraeolidea ianthina*. CT–ceratal tuft, OT–oral tentacle, Rh–rhinophore. Bar ~ 1.0 cm.

shrimp has been observed in the laboratory. *Berghia major* (Fig. 3) collected for this study fed on the swimming anemone *Boloceroides mcmurrichi* that contains zooxanthellae and is common in beds of *Acanthophora spicifera. Pteraeolidea ianthina* (Fig. 4) feeds on various species of hydroids. Observations of *P. ianthina* feeding in the laboratory, of its fecal remains, and of the nematocysts contained in its cnidosacs indicate that at least some of the prey species in the field are *Halocordyle distica* (= *Pennaria tiarella,* Cooke, 1977), a species of *Zanclea* (see Hastings, 1930) and unidentified species from the family Plumulariidae (hydroid taxonomic identifications by W. J. Cooke, University of Hawaii). None of these hydroids (nor others from *P. ianthina*'s habitat that were examined during an extensive survey by the author) contain zooxanthellae. No zooxanthella-containing hydroids, such as *Myrionema ambionenses* (Trench, 1979), are known to occur in Hawaiian waters (W. J. Cooke, pers. comm.). It should be noted that even though *P. ianthina* is often found in apparent association with the zooxanthellae-containing octocoral *Anthelia* (= *Sarcothelia*) *edmondsoni* (Kay, 1979), starved specimens of the aeolid would not feed on this coelenterate when placed with it in aquaria or fingerbowls. Starved *P. ianthina* would feed on the hydroid *Halocordyle distica* under similar circumstances.

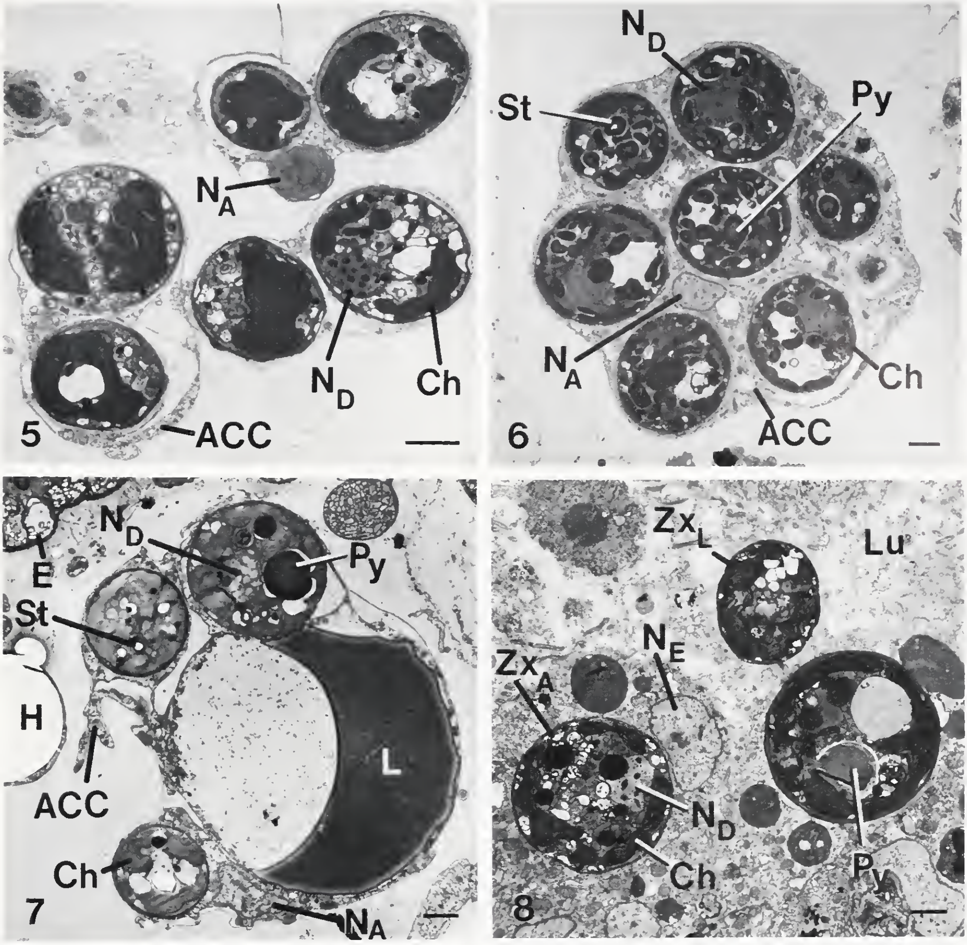
Location of algal symbionts

The egg masses, zygotes, and newly hatched larvae of the four species examined in this study do not contain zooxanthellae. Most of the zooxanthellae found in the adults of all four nudibranch species are intracellular and appear to reside within vacuoles in the cytoplasm of animal "carrier" cells (Figs. 5–8). Those algae that are not intracellular are found in the lumina of the digestive organs. In *P. ianthina* most of the "carrier" cells reside in a presumably protein matrix that forms a layer directly beneath the epidermis in all parts of the body (Fig. 7; also see 16, 17). It should be noted that this layer is not part of the hemocoel. In *Melibe pilosa* and *Melibe* sp. the algal cells, in their respective "carrier" cells, are distributed in a sparsely cellulated matrix that fills each ceras and the rest of the body in areas where specific organs or hemocoel are not located.

The animal cells containing zooxanthellae in all three species appear to have some association with the digestive gland. This association is particularly evident as it applies to extensions of this organ into the cerata. Intracellular zooxanthellae of *Berghia major* occur within epithelial cells of branches of the digestive gland in the cerata (Fig. 8). Algal cells are also found in an apparent proliferation of digestive gland cells that extends into the head region, oral veil, and rhinophores in larger specimens of this species. In all four nudibranch species, more than one algal cell may reside in a given "carrier" cell. Intracellular algal cell division stages have been observed in both species of *Melibe* and in *P. ianthina.*

Taxonomic identification of the algal symbiont

In all four nudibranchs, the vegetive intracellular stage of the alga is identical in morphology to *Symbiodinium* (= *Gymnodinium*) *microadriaticum* Freudenthal (Freudenthal, 1962; Kevin *et al.,* 1969; Taylor, 1974) (Figs. 5, 6, 7, 8). Algal cells artificialy separated from the animal tissue and kept in sea water or Provasoli's E.S. medium overnight form gymnodinoid zoospores like those described by Freudenthal (1962). Motile stages will also arise from zooxanthellae in host fecal pellets or decaying host tissues incubated under similar conditions in sea water.



FIGURES 5–8. Intracellular zooxanthellae in nudibranch ceratal tissues. ACC–animal carrier cell, Ch–chloroplast, N_A–animal carrier cell nucleus, N_D–dinoflagellate cell nucleus, St–starch granule, Py–pyrenoid.

FIGURE 5. *Melibe pilosa.* Bar = 2.0 μm.

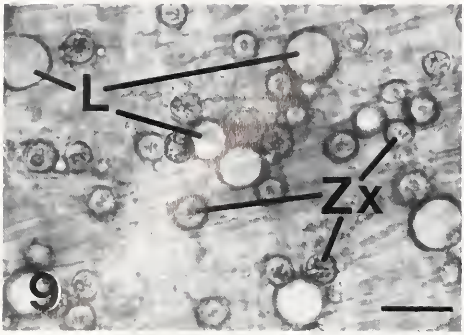
FIGURE 6. Unidentified *Melibe* sp. Bar = 2.0 μm.

FIGURE 7. *Pteraeolidea ianthina.* Algal cells and lipid droplet are within an animal carrier cell that resides in an acellular, presumably protein matrix below the epidermis. Note pyrenoid continuous with chloroplast. E–epidermis, H–hole caused by algal cell knocked out of plastic during sectioning, L–lipid droplet (partially extracted). Bar = 2.0 μm.

FIGURE 8. Intracellular zooxanthellae in presumed digestive cells lining ceratal extensions of the digestive gland in *Berghia major.* Lu–lumen of digestive gland extension into ceras, N_E–nucleus of animal cell in epithelial lining of central extension of digestive gland in ceras, Zx_A–zooxanthella in animal cell of epithilial lining of central extension of digestive gland in ceras, Zx_L–zooxanthella in lumen of central extension of digestive gland in ceras. Bar = 2.0 μm.

Lipid distribution in host tissues

Dense aggregations of refractile droplets are found associated with the intracellular zooxanthellae in adult *Pteraeolidea ianthina* (Fig. 9, also see 7). These droplets stain bright red in whole mounts stained with Sudan IV and green in Richardson's stained plastic sections. A few small droplets that stain red with Sudan IV are also found



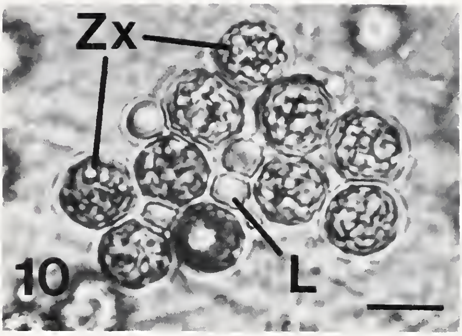


FIGURE 9. Unstained, refractile, lipid droplets in a ceras of *Pteraeolidea ianthina.* L–lipid droplet, Zx–zooxanthellae. Bar = 20 μm.

FIGURE 10. Unstained refractile lipid droplets associated with intracellular zooxanthellae in tissues of *Melibe* sp. L–lipid droplet, Zx–zooxanthellae. Bar = 10 μm.

associated with the intracellular zooxanthellae found in the tissues of adult *Melibe* sp. (Fig. 10). Adult *Melibe pilosa* and *Berghia major* lack refractile droplets.

The newly hatched veligers of all four species contain refractile droplets in their digestive diverticula. The presence of these droplets is particularly evident in newly hatched larvae of *Pteraeolidea ianthina* that contain refractile droplets throughout most larval tissues rather than just in the digestive diverticula. These refractile droplets also stain bright red with Sudan IV in larvae of *Melibe pilosa, Melibe* sp., and *P. ianthina* and green in sections of plastic-embedded larvae stained with Richardson's stain. Larvae of *Berghia major* were not stained.

Translocation of fixed carbon from symbiont to host

Starved adult specimens of *Melibe pilosa, Berghia major,* and *Pteraeolidea ianthina* survive longer in constant light than in constant dark (Table I). Comparative starvation tests were not performed with *Melibe* sp. Starved *M. pilosa* can survive a maximum of 29 days in constant light and 20 days in constant dark. Similar results were obtained for *B. major. Pteraeolidea ianthina,* on the other hand, survive as long as 192 days in constant light and up to 90 days in constant dark. All species tested decrease in mass during starvation. The mode and effects of this mass loss are most evident in *P. ianthina* due to its longer period of survival. Initially a slight increase in mass occurs, followed by a continuous decrease until death. Animals held in constant dark decrease in mass more rapidy than those held in constant light. After 19 days, *P. ianthina* that were initially the same size are still similar in length and appearance regardless of whether they have been starved in constant light or dark. When the same animals are examined after 61 days of starvation, those held in constant dark are considerably smaller than those held in constant light and in an obvious state of mortal decline. The presence of refractile droplets was followed in the cerata of one pair of starved *P. ianthina.* The specimen held in constant light lost all of its refractile droplets after 45 days of starvation and survived a total of 159 days. The animal starved in constant dark lost all of its droplets after 38 days of starvation and survived 74 days.

Host defecation of healthy and degenerate zooxanthellae

In all four species of nudibranchs examined, fecal pellets continue to be extruded throughout periods of starvation. Smears of *Melibe pilosa*'s fecal pellets examined

TABLE I

Effect of starvation in constant light or constant dark on survival in three species of nudibranchs symbiotic with Symbiodinium microadriaticum

Species	Duration of survival (days)	
	Constant light	Constant dark
Berghia major	37	22
Melibe pilosa[a]	25	19
	29	20
	29	20
	$\bar{X} = 28 \pm 2$	$\bar{X} = 20 \pm 1$
Pteraeolidea ianthina[a]	35[b]	74
	147	80
	159	90
	163[c]	81[c]
	192	82
	$\bar{X} = 165 \pm 19$	$\bar{X} = 81 \pm 6$

[a] Survival in constant light is significantly longer than in constant dark. *M. pilosa*, $P = 0.050$; *P. ianthina*, $P = 0.008$; Mann-Whitney U Test (Siegel, 1956).

[b] This animal did not appear to be healthy and was not included in calculation of mean survival time or significance.

[c] These animals were in the process of regenerating their body posterior to the fifth pair of ceratal tufts.

after one day of starvation contain nearly 100% healthy zooanthellae (Table II). Subsequent qualitative examination indicates that *M. pilosa*'s feces continue to be composed of nearly 100% healthy algal cells after several days of starvation. Fecal pellets of starved *Melibe* sp., *Berghia major*, and *Pteraeolidea ianthina* (*e.g.*, Fig. 11) contain large numbers of what are presumably degenerate algal cells. Ultrastructural examination of fecal pellets from starved specimens of *Melibe* sp. reveals both apparently healthy algal cells and algal cells at various stages of degeneration (Figs. 12–14). Counts of healthy and degenerate algal cells with the light microscope show that while about 97% of the algal cells in the feces of starved *M. pilosa* are healthy, over 96% of the algal cells from the feces of starved *Melibe* sp., *Berghia major*, and *Pteraeolidea ianthina* are degenerate (Table II). It is interesting to note that in fecal material from freshly collected (unstarved) *Melibe* sp. about 50% of the algal cells are healthy while after three days of starvation fecal pellets contain nearly 100% degenerate algal cells (Table II). This difference is significant at the $P = 0.001$ level.

The cerata of *Pteraeolidea ianthina* contain dark brown organs (Fig. 15) that are associated with the branches of the digestive gland that extend into the cerata and that contain both healthy and degenerate zooxanthellae. These are the only tissues in which degenerate algal cells were observed in *P. ianthina*. In sectioned material, these organs are continuous with both the sub-epidermal matrix that contains the "carrier" cells and their associated zooxanthellae and refractile droplets, and also with the extension of the digestive gland in each ceras (Figs. 16, 17). Algal cells can be seen within the organ's connection with the sub-epidermal matrix (Fig. 17). Zooxanthellae can sometimes be found within the lumen of the dark brown organ that is continuous with the lumen of the central digestive gland extension in each ceras (Fig. 16). Electron micrographs of sections of the organ show that intracellular zooxanthellae within it are either healthy or at different stages of degeneration. Dense-cored vesicles,

TABLE II

Appearance of zooxanthellae in fecal pellets from nudibranchs symbiotic with zooxanthellae

Species	n[a]	n[b]	# of days of starvation[c]	Zooxanthellae % healthy	Zooxanthellae % degenerate
Melibe pilosa	1	9	0	X̄ = 78 ± 29	X̄ = 22 ± 29
	5	10	1	X̄ = 97 ± 4	X̄ = 3 ± 4
Melibe sp.,					
Recently collected	6	10	0–1	X̄ = 47 ± 14	X̄ = 53 ± 14
After 3 days of starvation	6	10	3	X̄ = 3 ± 4	X̄ = 97 ± 4
Pteraeolidea ianthina	2	10	0	X̄ = 2 ± 4	X̄ = 98 ± 4
	1[d]	10	0	X̄ = 40 ± 39	X̄ = 60 ± 39
	7	10 or 20	1	X̄ = 2 ± 1	X̄ = 98 ± 1
	2	10	15	X̄ = 3 ± 6	X̄ = 97 ± 6
	2	10	22	X̄ = 3 ± 7	X̄ = 97 ± 7
Berghia major	1	11	?	X̄ = 2 ± 2	X̄ = 98 ± 2

[a] Number of fecal composition determinations. Each one representing a different animal.

[b] The number of equal sized areas in which the healthy and degenerate zooxanthellae for each fecal determination were counted.

[c] This is the same as the number of days since the animals were field collected.

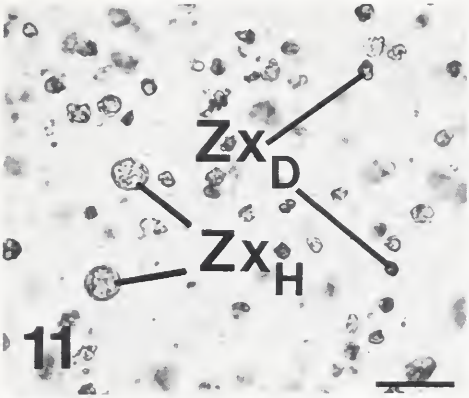
[d] The feces of this animal contained many hydroid perisarcal remnants suggesting that it had eaten recently and was not starved.

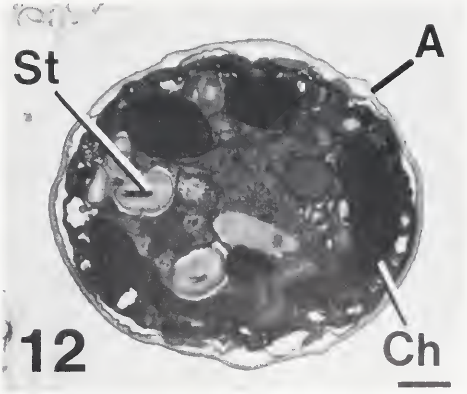
possibly lysosomes (Cook, 1980), are found in the animal cytoplasm surrounding the algal cells (Fig. 18).

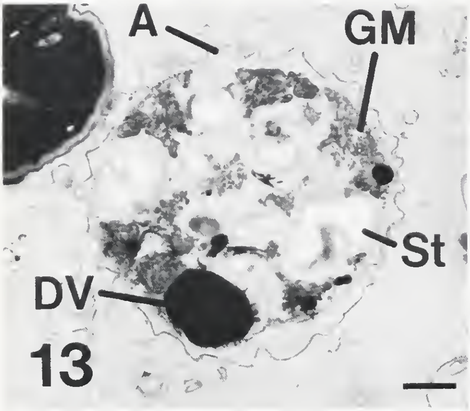
DISCUSSION

Location of algal symbionts in host tissues

As reported for numerous symbiotic coelenterates (Boschma, 1925; Muscatine, 1961; 1974; Mclaughlin and Zahl, 1966; Trench 1971a, b, c; Taylor, 1973; and others), the algal symbionts of *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* are contained within animal "carrier" cells that are apparently specialized for this purpose. A similar situation may exist to some extent in *Berghia major* in the anterior proliferation of symbiont containing cells found in larger specimens of this species; however, many of the zooxanthellae present in *B. major* are phagocytosed by and reside in cells of the digestive epithelium lining the ceratal extensions of the digestive gland. Similar to the conclusions of Rudman (1981a; 1982), for various symbiotic aeolid and arminacean nudibranchs, the animal "carrier" cells of the species examined in this study are associated with cells of the digestive gland and presumably are derived from cells of this linage. This conclusion is not unexpected in that the probable mode of infection, both at the historic inception of the symbiosis and at each new generation, is by phagocytotic uptake of ingested algal cells.







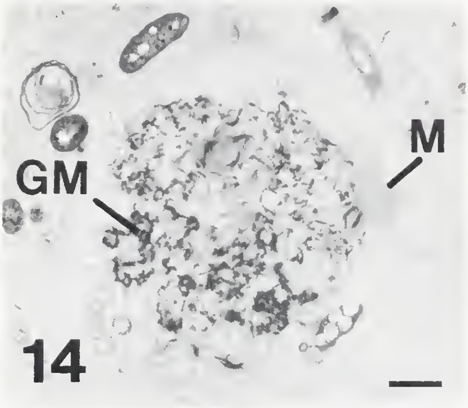


FIGURE 11. Light micrograph of squash of fecal pellet of *Pteraeolidea ianthina* showing presumably degenerate and healthy zooxanthellae. Zx_D–degenerate zooxanthellae, Zx_H–healthy zooxanthellae. Bar = 20 μm.

FIGURES 12–14. Healthy and degenerate zooxanthellae in fecal pellet of *Melibe* sp.

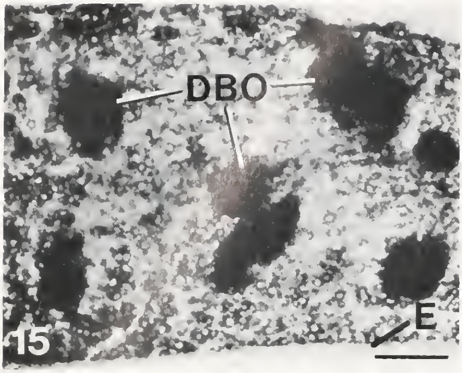
FIGURE 12. Morphologically intact, presumably healthy zooxanthella. Amphiesma is intact and regular, organellar structure is organized, and organelles and components are well defined. A–amphiesma, Ch–chloroplast, St–starch granule. Bar = 1.0 μm.

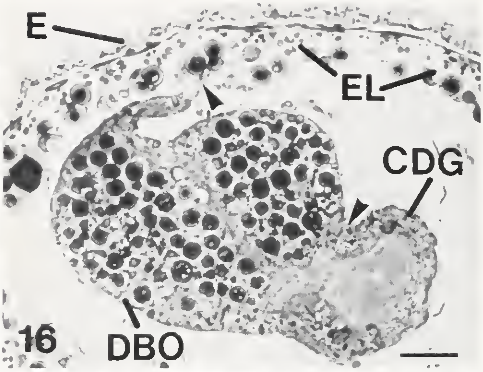
FIGURE 13. Degenerating zooxanthella. Amphiesma is highly irregular and beginning to break-up. A–amphiesma, DV–dense osmophilic vesicle, GM–granular matrix, St–starch granule. Bar = 1.0 μm.

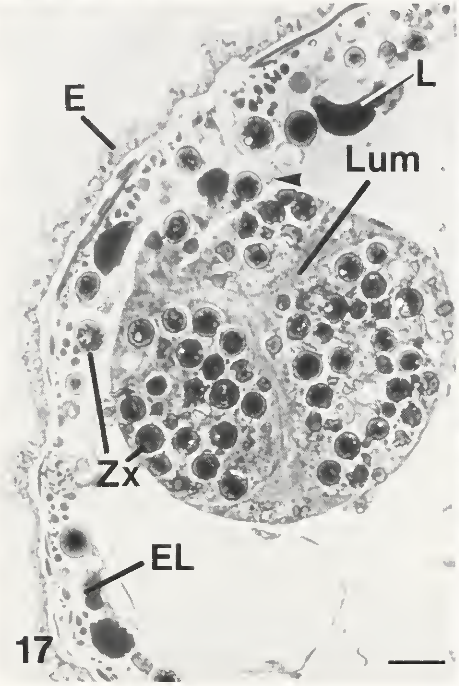
FIGURE 14. Probable membranous and granular remains of completely degenerated (digested?) zooxanthella. Granular matrix is similar to that within algal cell in Figure 13. Note myelin figures like those described by Trench (1974, p. 204) in degrading zooxanthella of *Zoanthus sociatus*. GM–granular matrix, M–myelin figure. Bar = 1.0 μm.

Translocation of nutrients from symbiont to host

The term "translocation" has been used in previous work to describe the leakage of fixed carbon from algal symbionts to the host tissues (Muscatine and Hand, 1958; Muscatine, 1967; Taylor, 1969a; Trench, 1971a, b, c; Goreau *et al.*, 1973; and others). The implication is that the mechanism for this leakage is a host-mediated transport or diffusion of fixed carbon across the amphiesma of healthy symbionts (Muscatine, 1967; Trench, 1971a, b, c). The results of starvation experiments carried out in the present study lead one to conclude that a similar mechanism for translocation functions in *Melibe pilosa*. Upon initial inspection of starvation results, a similar conclusion







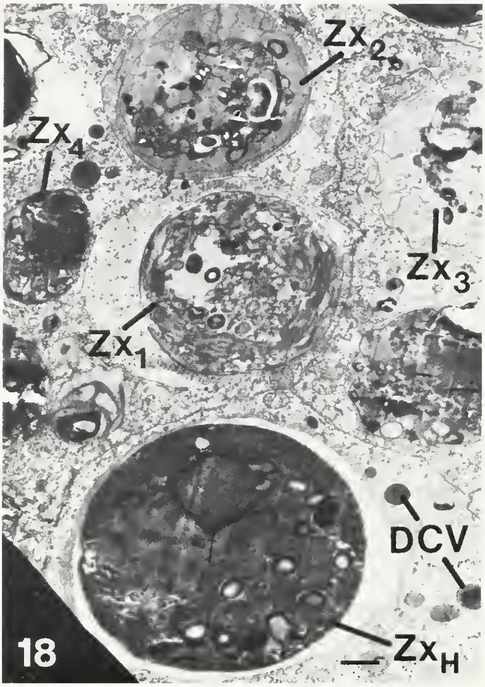


FIGURE 15. The dark brown organs that contain healthy and degenerate zooxanthellae as seen in a light micrograph of part of a whole ceras from *Pteraeolidea ianthina.* DBO–dark brown organs, E–epidermis of ceras, Bar = 200 μm.

FIGURE 16. Phase contrast micrograph of a transverse, Richardson's stained, plastic section of a ceras of *Pteraeolidea ianthina.* The connection of "dark brown organ" to a central extension of the digestive gland in the ceras and to the sub-epidermal layer containing the animal carrier cells and their associated zooxanthellae and lipid droplets can be seen. The lumen within the dark brown organ (see Figure 17) does not extend into the connection with the sub-epidermal layer. CDG–central extension of digestive gland into the ceras, DBO–"dark brown organ," E–epidermis of ceras, EL–sub-epidermal layer containing zooxanthellae and associated lipid droplets within animal carrier cells, Arrowheads–connections of "dark brown organ" with sub-epidermal layer and central extension of digestive diverticulum within ceras. Bar = 30 μm.

FIGURE 17. Phase contast micrograph of a transverse, Richardson's stained, plastic section of a ceras of *Pteraeolidea ianthina* showing zooxanthellae within the sub-epidermal layer's connection with the "dark brown organ." E–epidermis of ceras, L–lipid droplet, Lum–lumen within "dark brown organ." EL–sub-epidermal layer, Zx–zooxanthellae, Arrowhead–connection of "dark brown organ" with sub-epidermal layer (note zooxanthellae within this connective process). Bar = 20 μm.

might be reached for the other nudibranch species examined; however, since large numbers of degenerate algae are present in the feces of *Melibe* sp., *Pteraeolidea ianthina*, and *Berghia major*, it is possible that part or all of the fixed carbon assimilated by these hosts is obtained from the digestion (by host enzymes and/or autolysis) of algal cells (see below). The proportion of fixed carbon attributable to translocation via leakage and/or algal digestion remains to be determined.

Keeble's (1910) suggestion of translocation of "fat" from algal symbiont to host in the acoel flatworm *Convoluta roscoffensis* has recently been confirmed by Meyer *et al.* (1979). Investigations by Patton *et al.* (1977) and Blanquet *et al.* (1979) have demonstrated light dependent translocation of lipid or lipid precursors from symbiotic zooxanthellae to coelenterate hosts. Staining characteristics of the refractile droplets found associated with *Symbiodinium microadriaticum* in the algal "carrier" cells of adult *Melibe* sp. and *Pteraeolidea ianthina* identify the material composing the droplets as lipid. This suggests that translocation of lipid or lipid precursors occurs in these nudibranchs.

Lipid droplets reach very high densities in the tissues of *P. ianthina* and must comprise a substantial energy reserve. Similar high concentrations of lipid in the larvae of this nudibranch [higher concentrations than are found in other opisthobranch larvae (Kempf, 1982)] suggest that some of this lipid is utilized in reproduction.

Digestion of Symbiodinium microadriaticum *by nudibranch hosts*

Digestion of *Symbiodinium microadriaticum* by the invertebrate host has been suggested for tridacnid clams (Yonge, 1936; Fankboner, 1971) and the coelenterates *Astrangia danae* (Boschma, 1925) and *Phyllactis flosculifera* (Steele and Goreau, 1977). Trench (1980) believes that there is not sufficient evidence to support hypotheses of the occurrence of ". . . *in situ* intracellular or extracellular digestion of symbiotic dinoflagellates" in these species. He suggests that previous reports of zooxanthella digestion by hosts may have been due to observations of senescent algal cells that had undergone autolysis (Trench, 1974, 1980). Recently Trench *et al.* (1981) have offered further evidence in support of the autolytic degradation hypothesis for tridacnid species.

The following observations of degenerate algal cells in *Berghia major*, *Melibe* sp., and *Pteraeolidea ianthina* suggest that these three nudibranchs take an active part in the breakdown of at least some of their algal symbionts: a) the degenerate algal cells that are observed in the feces of these species, b) the near-absence of pycnotic algal cells in the feces of *M. pilosa* while the feces of the other three species examined contain a high percentage of degenerate algal cells, c) the increase in the proportion of degenerate algal cells during starvation of *Melibe* sp. [This is the opposite of what has been observed in *Tridacna gigas* (Trench *et al.*, 1981)], d) the presence of an organ in *P. ianthina* the contents and anatomical position of which indicate its possible function as an algal digestive organ (see below), and e) the extended survival that continues far beyond the point at which algal-associated lipid reserves are depleted when *P. ianthina* is starved in constant light or constant dark. While the research

FIGURE 18. Electron micrograph of a morphologically intact and presumably healthy zooxanthella and different stages of zooxanthella degradation (presumably digestion) within one of the "dark brown organs" found in the cerata of *Pteraeolidea ianthina*. Dense core vesicles, possibly lysosomes, can be seen in the cytoplasm of cells containing zooxanthellae. DCV–dense core vesicle, Zx_H–presumably healthy zooxanthella, Zx_1, Zx_2, Zx_3, Zx_4–stages in the degradation (digestion?) of zooxanthellae in a "dark brown organ" (Zx_3–note apparent breakup of algal wall and plasmalemma). Bar = 1.0 μm.

reported in this study does not conclusively demonstrate that these species enzymatically digest *S. microadriaticum,* this would appear to be one of only two possibilities, the other being that these hosts in some way affect and regulate the autolytic degradation of algal cells.

It is suggested that *Melibe* sp. *Pteraeolidea ianthina* digest some of their algal symbionts, consequently regulating algal density in their tissues (see Taylor, 1969b; Muscatine and Pool, 1979) and at the same time obtaining (via digestive translocation) symbiont-derived nutrients over and above those provided by leakage translocation of fixed carbon and lipid or lipid precursors from healthy algal cells. The increase in the proportion of degenerate algal cells in the feces of *Melibe* sp. shortly after the beginning of starvation suggests that at least this nudibranch can either regulate the rate of algal digestion and perhaps the mitotic rate of its symbionts to its nutritional advantage or that it can control the rate of expulsion of healthy algal cells in its feces. In adult *P. ianthina,* the algal cells, and perhaps lipid droplets, would appear to be removed from their residence in the subepidermal layer and transferred through its connection with the "dark brown organ" to digestive cells within that organ. The indigestible remains of algal cells and a few healthy symbionts would then be exocytosed into the lumen of the organ and subsequently passed out of the animal in its feces. It should be noted that while it is possible that *Berghia major* can digest established symbionts residing in its tissues, it seems more likely that this nudibranch digests mostly excess algal cells derived from its prey as has been reported for *Cuthona poritophages* by Rudman (1981b).

Infection of nudibranch hosts with Symbiodinium microadriaticum

Since the newly hatched larvae of all the nudibranchs examined in this study lack algal symbionts, one must conclude that the symbiosis is re-established in each new generation of host organisms. In *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina,* nudibranchs that do not feed on prey symbiotic with zooxanthellae as adults, it seems probable that this reinfection occurs in one or more of the following ways: 1) by inadvertant ingestion of *Symbiodinium microadriaticum* during larval or post-metamorphic feeding (LaBarbara, 1975; Fitt and Trench, 1981), 2) by juvenile feeding on a food source that contains the symbionts and is different than the prey of the adult (juvenile feeding on a food source different than that of the adult has been reported for the non-symbiotic nudibranch *Doridella obscura* by Perron and Turner, 1977), 3) by ingestion of the symbiont that is accomplished by some sort of perceptual mechanism, such as chemotaxis (Kinzie, 1974; Fitt and Trench, 1983), that acts to bring host and symbiont together.

Kempf (1982) has shown that the digestive cells of larvae of *M. pilosa* and possibly *P. ianthina* are capable of phagocytosing the unicellular alga *Pavlova (Monochrysis) lutheri.* This phagocytic potential offers a mechanism that would allow larval inception of the symbiosis in these species. The lack of algal cells in pre-metamorphic larvae of *B. major* and the presence of algae in the tissues of newly metamorphosed juveniles after feeding on adult prey (unpub. pers. obs.) suggest that each new generation of this nudibranch is re-infected with symbiotic algae obtained from its prey after metamorphosis.

Inception and evolution of nudibranch-zooxanthellae symbioses

The evolution of intracellular symbioses (Richmond and Smith, 1979; Smith, 1979; Taylor, 1979) and algal-invertebrate symbioses in particular (Trench, 1979,

1980) have been recently reviewed. Trench (1980) pointed out that examination of existing symbioses will allow insight into the evolutionary origin(s) of the many possible levels of host-symbiont integration, but that one must take care not to assume that patterns seen among extant associations mimic evolutionary pathways. In the case of nudibranch symbioses with zooxanthella(ae?), Rudman (1982) has recently speculated that these associations are of a "secondary" nature and arose through the mechanism of a selective opportunity afforded by the nudibranch hosts feeding on prey symbiotic with the alga(ae?). This hypothesis was based on his observation that nearly all the nudibranch species he examined were known to feed on such organisms. The present study has shown that adult *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina* maintain an intracellular and possibly mutualistic symbiosis (however, see below) with the zooxanthella *Symbiodinium microadriaticum.* That these nudibranchs do not feed on prey containing these symbionts suggests that the evolutionary inception of their symbiotic association was not of a secondary, opportunistic nature. Thus, it appears that there are at least two pathways by which nudibranch-zooxanthella symbioses could have been established; either 1) via the opportunity afforded by the host feeding directly on prey that are symbiotic with this dinoflagellate (Rudman, 1982) or 2) by inadvertant (at least on the part of the host) ingestion of the symbiont while feeding.

In the first instance, ample opportunity for the selection of a symbiosis between the potential host and *Symbiodinium microadriaticum* would occur as the potential host feeds on organisms symbiotic with the alga. Additionally, the alga might be predisposed to a symbiotic relationship with the potential host by virtue of such an association existing between the alga and the nudibranch's prey.

If, as appears to have been the case for *Melibe pilosa, Melibe* sp., and *Pteraeolidea ianthina,* the potential host's only exposure to *Symbiodinium microadriaticum* was by inadvertent ingestion, and therefore possibly rare, the mechanism(s?) by which a mutualism might have been established are not as clear. A predisposition of *Symbiodinium microadriaticum* to symbiotic associations, as mentioned above, may have been a factor; however, perhaps the suggestion of Goetsch (1924) and Goetsch and Scheuring (1927) also offers a plausible answer. They suggested that mutualistic relationships could have arisen from symbioses that were initially beneficial to the symbiont, but detrimental to the host (*i.e.,* of a parasitic nature). In such associations that did not become extinct because of mortal virulence of the symbiont, selection for host immunity, or some other cause, it seems likely that selection would tend to favor modification of the association such that the host benefits to the symbiont would be maintained or enhanced and the pathological effects of the symbiont on the host would be decreased. Under such circumstances it is not hard to envision selection leading to the establishment of a mutualism.

It has been suggested above that the nudibranch symbioses examined in this investigation might be mutualisms; however, the apparent digestion of symbionts by three species of nudibranchs leads one to ask if their symbioses should really be viewed as mutualistic. Rather than interactions involving mutual benefits, it appears that such hosts have "gained the upper hand" in the association and now hold the algal symbiont in bondage. It is, of course, possible that the clonal nature of the symbiont may offset the apparent disadvantages of digestion by the host, the alga asexually reproducing far more copies of its genome than the host digests.

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VARIATION IN THE DIAPAUSE RESPONSE OF *LABIDOCERA AESTIVA* (COPEPODA: CALANOIDA) FROM DIFFERENT LATITUDES AND ITS IMPORTANCE IN THE EVOLUTIONARY PROCESS

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Abstract

Labidocera aestiva were collected from three distantly separated sites along the Atlantic coast of the United States. Their offspring were reared in the laboratory under several combinations of photoperiod and temperature to assess the diapause response and genetic similarity of the populations. The results indicate that *L. aestiva* is not genetically homogeneous throughout its range. Differences in the percentage of subitaneous and diapause eggs produced by laboratory-reared and field-collected populations reflected the unique combination of environmental conditions occurring in the geographic regions from which the animals originated. In the Virginian province where planktonic stages of the species are seasonally present, diapause egg production is triggered by short day length photoperiods and cool temperatures. On the other hand in the Carolinian province where the planktonic stages tend to occur year-round diapause eggs are rarely produced by either field-collected females or their laboratory-reared offspring. This suggests that most of these animals lack the genetic capacity to produce diapause eggs. Diapause may lead to the reproductive isolation of populations if gene flow is reduced or prevented owing to their separation on a temporal scale.

Introduction

It has long been recognized that many marine planktonic organisms have very broad spatial distributions which often encompass an extensive latitudinal gradient (see discussions in Ekman, 1953; Raymont, 1963; Fleminger, 1975; Van der Spoel and Pierrot-Bults, 1979). This pattern has been attributed largely to their potential for passive dispersal across great distances in ocean currents. Despite evidence of phenotypic differences in morphological, behavioral, and/or physiological traits between populations from distant areas, marine biologists have argued that planktonic species are genetically homogeneous due to the potential for considerable panmixia (*i.e.*, random interbreeding of populations). Consequently, the observed phenotypic differences have often been ascribed to environmental modification. Progress in determining the basis for such variation has been hindered by difficulties encountered in obtaining, maintaining, and rearing planktonic species for genetic studies (Paffenhöfer and Harris, 1979). However, some insight into the genetic structure of planktonic populations has been gained from electrophoretic studies (*e.g.*, Manwell *et al.*, 1967; review by Gooch, 1975; Ayala and Valentine, 1979). An important outcome of these studies is that planktonic species are no longer regarded as necessarily being genetically homogeneous. A weakness with this approach, however, is that the adaptive significance of different electromorphs (*i.e.*, mobility variants revealed by electrophoresis) is often

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unknown. Thus, their importance in the evolutionary process and the formation of new species is difficult to assess. Studies that provide insight into the genetic divergence of traits of obvious adaptive significance should lead to a better understanding of the mechanism(s) which enable species to cope with environmental change, the potential importance of emigration in the re-population of foreign areas, and the evolutionary steps that are basic to speciation.

Labidocera aestiva is a calanoid copepod reported to occur in North American coastal waters from the Gulf of St. Lawrence to the Gulf of Mexico (Grice, 1956; Fleminger, 1957; Deevey, 1960; Cronin *et al.,* 1962; Anraku, 1964; Van Engel and Tan, 1965; Bowman, 1971; Turner *et al.,* 1979). In the northern portion of the range (*e.g.,* Vineyard Sound, Massachusetts) it is seasonally present in the plankton. Adults appear in mid-June and give rise to a series of generations stemming from subitaneous eggs which hatch within a few days of extrusion. By mid-December nauplii, copepodids, and adults disappear from the plankton, but the species maintains its presence in the area in the form of benthic diapause eggs (Marcus, 1979). Diapause eggs are produced during the fall, but do not hatch until the following spring. Thus, the first generation of animals that appears in June is derived from these diapause eggs. The ecological significance of the benthic diapause stage is that it enables survival of individuals during the periods that cannot be tolerated by the planktonic stages. The onset of diapause prevents the waste of partial generations. The termination of diapause synchronizes the life cycle of the species with the environmental regime so that development, growth, and reproduction are optimized. By rearing the offspring of females collected in Vineyard Sound, Massachusetts in the laboratory it has been shown that the type of egg produced by a female is triggered by a temperature-compensated photoperiodic response (Marcus, 1980a, b; 1982a, b). Short day lengths and cold temperatures are most effective in the induction of diapause eggs, whereas long day lengths and warm temperatures are more effective in the induction of subitaneous eggs. There is some variation among female *L. aestiva* in the particular combination of photoperiod and temperature which effectively triggers diapause egg production, and this variation helps to ensure survival of the species despite year to year fluctuations in environmental conditions.

The seasonal cycling of environmental conditions varies considerably along the latitudial gradient inhabited by *L. aestiva* resulting in longer periods of occurrence of populations in the plankton as one moves in a southerly direction. In Florida, where seasonal environmental fluctuations are small, the growth, development, and reproduction of *L. aestiva* in the plankton continues throughout the year (Bowman, 1971; Blades-Eckelbarger, pers. comm.). Published data on the temporal distribution pattern of the species along the coast (Deevey, 1960; Cronin *et al.,* 1962; Bowman, 1971; Lonsdale and Coull, 1977) indicates that the timing and/or expression of diapause may differ between populations. These differences could reflect either environmental modification of the phenotype or genetic differentiation.

This study was designed therefore to determine and compare the diapause response of populations of *L. aestiva* from different locations along the latitudinal gradient which makes up its range on the Atlantic coast of North America. The study used two approaches: 1) determination of the type(s) of eggs produced under a range of photoperiodic and temperature conditions by laboratory-reared offspring of different geographic heritages and 2) determination of the type(s) of eggs produced by freshly caught females from the field. The results indicate that the species is spatially subdivided into populations which differ in the expression of certain diapause traits and that these differences reflect genetic divergence. This differentiation exists despite the apparent potential for extensive transport and mixing of the planktonic stages in the ocean currents. Diapause thus represents an adaptive response which may be of fundamental importance in the evolutionary process of speciation of some marine planktonic organisms.

MATERIALS AND METHODS

Labidocera aestiva were collected from the plankton during a three-year period from September, 1979, to August, 1982, at four locations along the East coast of the United States: Delaware Bay, Delaware; Wachapreague Inlet, Virginia; Beaufort Inlet, North Carolina; and Fort Pierce Inlet, Florida. Samples were collected with 240 μm mesh plankton nets (mouth diameter either 0.25 or 0.75 m). The nets were either towed for five to ten minutes off a boat or in the case of some collections from Beaufort, the net was suspended off the Pivers Island bridge for several hours. The surface water temperature was determined at the time of each collection. In the laboratory adult *L. aestiva* were removed from the samples using a wide-mouth pipette and placed in large buckets containing filtered (5 micron) sea water. The contents were aerated and *Gymnodinium nelsoni* or *Artemia* nauplii were added to the containers as food. Within one to three days the animals were transported back to Woods Hole in insulated containers for experimental manipulation. In Beaufort and Fort Pierce, laboratory facilities were available at the Duke Marine Laboratory and Harbor Branch Foundation respectively, which made it possible to do the experimental analyses of the field-collected females within 24 hours of collection. If sufficient numbers of *L. aestiva* were present in the plankton samples, then some females were removed from the buckets, placed into small dishes containing filtered sea water and *Artemia* nauplii, and incubated overnight at room temperature (*i.e.*, 20°C–23°C). The following day eggs were collected and transferred to another dish containing filtered sea water. The eggs were examined after 2 to 3 days to determine the number that had hatched. After five days unhatched eggs were placed into glass screw-capped jars containing filtered sea water and chilled at 5°C in a refrigerator. These jars were transported back to Woods Hole and subsequently maintained at 5°C. After chilling for a minimum of 4 weeks the jars were warmed at 19°C or 25°C and the number of eggs that hatched after this treatment was determined. The adults which were transported back to Woods Hole were maintained for as long as possible in the laboratory, in either buckets or 19 liter glass carboys. The buckets were provided with airstones, whereas the carboys were mounted on a mechanical rotator. In both cases the animals were provided with a mixed diet of four dinoflagellates: *Gymnodinium nelsoni, Prorocentrum micans, Gonyaulax polyedra,* and *Scrippsiella trochoideum* (previously referred to as *Peridinium trochoideum*). These field-collected animals were used to initiate most experimental cultures. Females were transferred from the buckets or carboys into dishes containing filtered sea water. They were fed *Gymnodinium nelsoni* and incubated overnight at 19°C. The next day eggs were collected and incubated for two more days at 19°C. Hatched nauplii were placed into 19 liter glass carboys and reared to reproductive maturity as described previously for animals from Vineyard Sound (Marcus, 1980a, b). The carboys were mounted on a mechanical plankton rotator in an environmental chamber equipped with light cycling and temperature controls. The responses to nine different combinations of temperature (14.5°C, 18.0°C, 24.0°C) and photoperiod regime (18L–6D, 12L–12D, 8L–16D) were examined as was done previously for the animals from Vineyard Sound (Marcus, 1980a, 1982b). The temperature fluctuated ±1°C throughout the 24-hour cycle. Delaware Bay animals were also reared at one additional set of environmental conditions (*i.e.*, 10L–14D at 18°C). Due to equipment problems, the temperature level occasionally exceeded the set point by several degrees. Such failures were remedied within 24 hours and only rarely resulted in the mass mortality of animals in the experimental carboys. On these occasions new cultures were started to obtain the necessary data. Breakdowns also affected some of the algal cultures used for feeding so that periodically the diet had to be modified to include more or less one of the food species. Every effort was made to initiate experiments from the eggs of field-collected adults or the second generation offspring (to reduce the possible effects of selection due to laboratory conditions). This was not always possible due to the difficulties encountered in obtaining animals from the different sites. Thus a few experiments were started from the eggs of third generation animals, and one (D11) was based on those of fourth generation animals. For all experiments the same schedule of feeding, transfer of animals to clean sea water, and egg collection reported by Marcus (1980a, 1982b) was followed in this study. Also the types of eggs which were produced were determined for each set of conditions according to the criteria of Marcus (1982b).

RESULTS

Seasonal field data: adult abundance and temperature conditions

The location of the four sites sampled in this study and the Vineyard Sound site sampled by Marcus (1979, 1980a, 1982a, b) are shown in Figure 1. *L. aestiva* was first obtained from Fort Pierce in September, 1979. Thereafter animals were obtained in April, 1980, 1981; January, 1981; February, 1981, 1982; August, 1981; and September, 1981. The surface water temperature ranged from 17°C in January to 27°C in August and September. Although the spatial distribution of *L. aestiva* was somewhat patchy in this area (*i.e.*, sometimes a tow would have no *L. aestiva*, yet moving to another spot yielded hundreds to thousands) adults were always abundant. This is a qualitative description of the samples as the methods of collection were not designed for a quantitative analysis. On the other hand, *L. aestiva* were not so readily obtained in Beaufort. A sampling trip in April 1981 yielded only a few adults. Adults were successfully collected in July, 1981, November, 1981, and August, 1982, but never in the quantities encountered at Ft. Pierce. The surface water temperature ranged from 13°C in April to 30°C in July. Considerable difficulty was encountered in obtaining *L. aestiva* in Wachapreague Inlet. Numerous tows taken in November, 1981, yielded no animals. The water temperature was 11°C. In June, 1982, *L. aestiva* adults were still not abundant, although some copepodites were present. The water temperature was 20°C. In July, 1982, adults again were not obtained. Thus, in July, 1982, Delaware Bay was chosen as a new study site. Several tows were taken and by pooling their contents hundreds of females were obtained. The water temperature at this time was 22°C. We returned to this location in August, 1982, but were unable to obtain sufficient animals. Thus all experimental manipulations for Delaware Bay were based on the one collection of July, 1982.

Egg production by field-collected and laboratory-reared females

Offspring of Delaware Bay, Beaufort, and Fort Pierce heritages were successfully reared through reproductive maturity under all of the combinations of photoperiod and temperature tested. The percentages of eggs that hatched after the initial incubation at 25°C and that which hatched after prolonged exposure to 5°C are shown for each carboy in Tables I, II, and III. F values derived from an analysis of variance for the arc-sin transformed data indicate no significant difference between the means of replicate carboys for 23 of the 28 combinations tested. The average percentages of

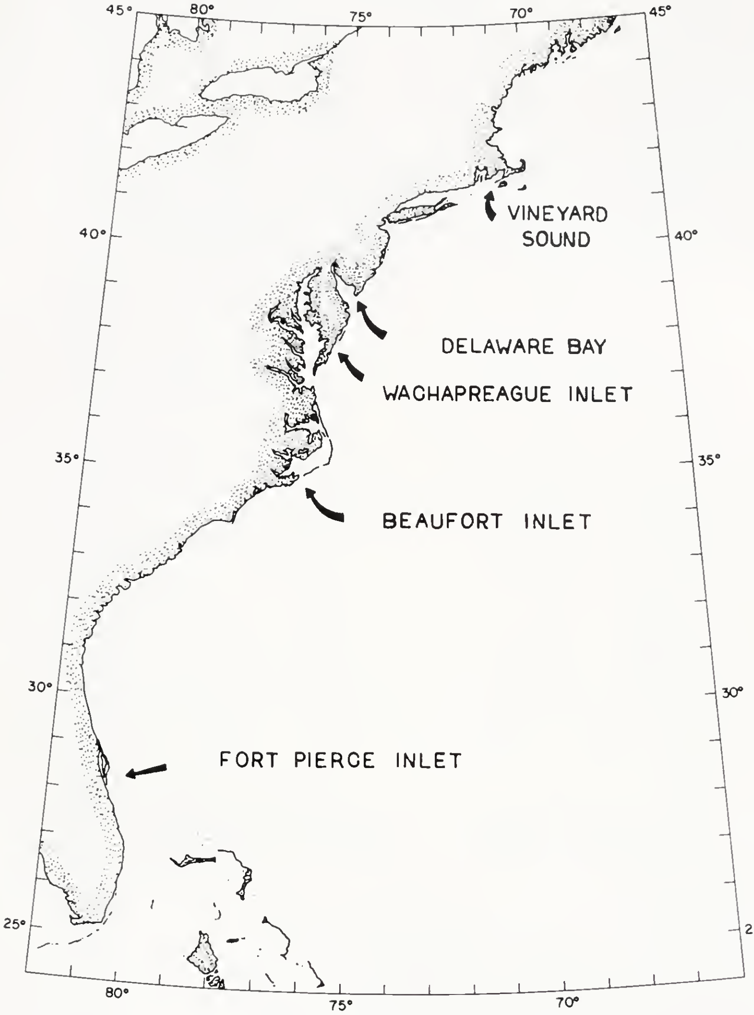
45°
80°
75°
70°
45°
VINEYARD SOUND
40°
40°
DELAWARE BAY
WACHAPREAGUE INLET
35°
35°
BEAUFORT INLET
30°
30°
FORT PIERCE INLET
25°
25°
80°
75°
70°

FIGURE 1. Location of study sites.

subitaneous, diapause, and non-viable eggs at each photoperiodic and temperature regimen are summarized in Figure 2.

Laboratory-reared females of Ft. Pierce heritage rarely produced diapause eggs (less than 2.0% of all eggs produced) even under the short day length and cool temperatures that favor their production by Vineyard Sound animals (data of Marcus, 1980a, 1982b). Similarly, diapause eggs were rarely obtained from the field-collected specimens.

Animals of Beaufort heritage responded differently. Diapause eggs were obtained from laboratory-reared females under short day length conditions, but not consistently, and not in large numbers (*e.g.,* 6.0%). Diapause eggs (*ca.* 22.0%) were produced by females collected in the field during November, 1981.

The diapause response of laboratory-reared females of Delaware Bay heritage was quite different from the Beaufort and Ft. Pierce populations. Diapause eggs were

TABLE I

*Percent hatch of eggs produced by females reared at 13.5°–15.5°C under 8L–16D, 12L–12D, or 18L–6D**

	DELAWARE			BEAUFORT			FT. PIERCE		
Photo-period	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C
8L–16D	D1^{1}	27.8 ± 8.2	80.8 ± 10.1	B1^{3}	90.7 ± 2.5	—	F1^{1}	79.5 ± 2.4	—
	D2^{1}	27.2 ± 9.1	79.6 ± 8.4	B2^{2}	86.1 ± 5.0	—	F2^{1}	86.4 ± 4.4	—
	D3^{1}	27.4 ± 7.7	84.3 ± 10.9						
X		27.5 ± 0.2	81.5 ± 1.4		88.4 ± 2.3	—		83.0 ± 3.5	
12L–12D	D4^{3}	80.3 ± 8.3	40.0 ± 25.1	B3^{1}	78.5 ± 8.2	13.8 ± 8.0	F3^{1}	85.8 ± 2.2	—
	D5^{3}	84.8 ± 2.6	16.0 ± 11.3	B4^{2}	72.7 ± 10.2	0.0	F4^{3}	87.9 ± 1.9	0.0
				B5^{2}	74.1 ± 13.7	0.0			
X		82.6 ± 2.3	22.0 ± 6.8		75.1 ± 1.8	4.6 ± 4.6		86.9 ± 1.1	
18L–6D	D6^{2}	79.3 ± 6.4	0.0	B6^{3}**	86.8 ± 2.2	1.3 ± 2.3	F5^{1}	81.8 ± 1.6	—
	D7^{2}	79.5 ± 6.6	0.0	B7^{3}**	76.2 ± 1.3	2.8 ± 3.8	F6^{2}	84.9 ± 2.5	3.0 ± 2.7
				B8^{1}**	88.3 ± 3.8	3.3 ± 6.5	F7^{2}	87.4 ± 3.3	6.0 ± 4.6
X		79.4 ± 0.1	0.0		83.8 ± 3.8	2.4 ± 0.6		84.7 ± 1.6	4.5 ± 1.5

* Eggs that did not hatch within 4–5 days at 25°C (initial) were incubated in jars at 5°C. The final hatch of these eggs at 25°C is indicated. D = Delaware, B = Beaufort, and F = Fort Pierce. Superscripts refer to 1, 2, 3, or 4 generation offspring. X = mean ± S.E.

** Significant difference at .05 level.

TABLE II

*Percent hatch of eggs produced by females reared at 17.0°–19.0°C under 8L–16D, 10L–14D, 12L–12D, or 18L–6D**

	DELAWARE			BEAUFORT			FT. PIERCE		
Photo-period	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C
8L–16D	$D8^1$	20.0 ± 2.9	84.0 ± 7.8	$B9^1$	90.3 ± 3.3	—	$F8^2$	84.5 ± 4.5	9.0 ± 15.6
	$D9^1$	23.7 ± 7.0	78.3 ± 14.2				$F9^2$	80.3 ± 3.1	2.3 ± 4.0
	$D10^1$	19.4 ± 6.8	80.6 ± 7.6						
X		21.0 ± 1.3	81.0 ± 1.7					82.4 ± 2.1	5.7 ± 3.3
10L–14D	$D11^4$	44.3 ± 11.4	83.0 ± 6.4						
	$D12^2$	54.0 ± 10.4	59.8 ± 13.3						
X		49.2 ± 4.9	71.4 ± 11.6						
12L–12D	$D13^3$	93.3 ± 1.3	—	$B10^2$	78.8 ± 3.4	9.0 ± 10.2	$F10^1$**	77.8 ± 4.3	—
	$D14^3$	91.8 ± 3.4	—	$B11^2$	79.6 ± 3.1	24.0 ± 0.0	$F11^2$**	92.7 ± 2.1	15.0 ± 21.2
X		92.6 ± 0.8	—		79.2 ± 0.4	16.5 ± 7.5		85.3 ± 7.5	
18L–6D	$D15^2$	93.4 ± 2.5	—	$B12^1$	88.7 ± 2.7	—	$F12^1$**	71.7 ± 2.9	1.3 ± 2.5
	$D16^2$	95.8 ± 1.3	—	$B13^1$	85.8 ± 2.5	—	$F13^1$**	87.7 ± 4.0	0.0
X		94.6 ± 1.2	—		87.3 ± 1.5	—		79.7 ± 8.0	0.6 ± 0.6

* Refer to Table I for explanation of hatch data and symbols.
** Significant difference at .05 level.

TABLE III

*Percent hatch of eggs produced by females reared at 23.0°–25.0°C under 8L–16D, 12L–12D, or 18L–6D**

	DELAWARE			BEAUFORT			FT. PIERCE		
Photo-period	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C	Carboy no.	Initial 25°C	Final 5°C
8L–16D	D17^{3}**	69.0 ± 11.0	81.4 ± 11.3	B14^{1}	74.8 ± 16.0	57.3 ± 29.1	F14^{2}	86.5 ± 7.3	—
	D18^{3}**	83.3 ± 2.6	24.8 ± 14.3	B15^{1}	81.3 ± 3.7	54.7 ± 10.1	F15^{3}	86.3 ± 5.1	7.5 ± 10.6
				B16^{1}	82.1 ± 4.8	10.0 ± 17.3	F16^{3}	80.8 ± 11.0	0.0
				B17^{1}	87.5 ± 0.1	0.0			
X		76.2 ± 7.2	53.1 ± 28.3		81.4 ± 2.6	30.5 ± 14.9		84.5 ± 1.9	3.8 ± 3.8
12L–12D	D19^{2}	76.3 ± 11.2	84.2 ± 13.9	B18^{2}	90.7 ± 3.2	—	F17^{1}	89.0 ± 1.0	—
	D20^{2}	80.8 ± 11.1	89.0 ± 6.0	B19^{2}	87.7 ± 1.5	—	F18^{1}	89.7 ± 4.2	—
X		78.6 ± 2.3	85.6 ± 1.4		89.2 ± 1.5	—		89.4 ± 0.4	—
18L–6D	D21^{2}**	79.0 ± 4.0	2.0	B20^{2}	92.1 ± 3.8	—	F19^{1}	88.4 ± 1.0	—
	D22^{2}**	62.3 ± 2.1	0.0	B21^{3}	89.0 ± 2.1	0.0	F20^{1}	83.6 ± 4.5	—
							F21^{1}	81.1 ± 5.8	—
X		70.7 ± 8.4	0.0		90.6 ± 1.6	—		84.4 ± 2.1	—

* Refer to Table 1 for explanation of hatch data and symbols.
** Significant difference at .05 level.

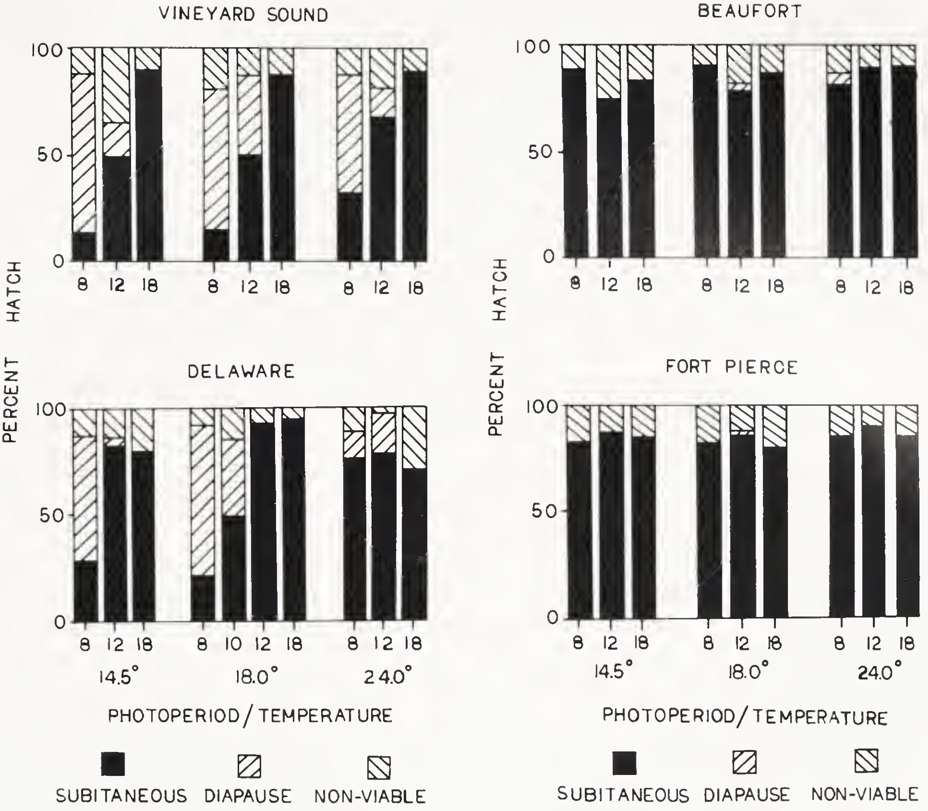

FIGURE 2. Subitaneous, diapause, and non-viable egg production (percent) by females reared under 8L–16D, 10L–14D, 12L–12D, or 18L–6D at 14.5°C, 18.0°C, or 24.0°C (±1.0°C). a) Vineyard Sound and Delaware Bay, b) Beaufort and Fort Pierce. Data for Vineyard Sound from Marcus (1980a, 1982b).

consistently produced by these females. Females that were collected from the field in July did not produce diapause eggs. For Delaware Bay animals reared in the laboratory, short day lengths were most effective in the induction of diapause egg production. Although a 12L–12D photoperiod at 18°C did not result in a marked decline of subitaneous egg production compared to 18L–6D, a decline in subitaneous and an increase in diapause egg production was obtained by rearing the animals at 10L–14D at this temperature. The percentage of non-viable eggs produced ranged from 3.0% to 29.3% for the entire study, but most values were less than 15.0%.

DISCUSSION

The results of this study demonstrate that *L. aestiva* is not genetically homogeneous throughout its range, but rather is composed of distinct populations genetically adapted to local environmental conditions. Within each region the critical photoperiod for diapause induction expressed by the population has been adjusted by natural selection to maximize growth of the species. Due to latitudinal variation in climatic (*e.g.*, temperature, Bumpus, 1957) and day length conditions (U. S. Dept. of Commerce, 1983), disruptive selection has promoted differentiation of populations along the north-south gradient. This divergence has occurred despite the potential for gene flow via transport of planktonic stages in ocean currents.

The general lack of diapause egg production by females collected from Ft. Pierce waters as well as from their laboratory-reared offspring strongly suggests that most of these animals do not have the genetic capacity to produce diapause eggs. This seems reasonable since diapause eggs would not be as important for survival in this region owing to the species year-round presence in the plankton (P. Blades-Eckelbarger, pers. comm.). The basis of the significant variation that exists among the replicate carboys at 18°C, 12L–12D and 18°C, 18L–6D is not known. In both cases one carboy yielded unexpectedly lower subitaneous egg production (77.8% and 71.7%, respectively) than the other duplicate culture (92.7% and 87.7%, respectively). However, the replicates for each set were reared at the same time and were exposed to the identical environmental regimen and diet. The cultures were also started from the eggs of field-collected adults or their first generation offspring so that inbreeding is an unlikely cause.

In the Beaufort region *L. aestiva* adults have been reported year-round as well, although their abundance fluctuates seasonally (Sutcliffe, 1950; Bowman, 1971). The species is widespread across the shelf in the winter and spring, but restricted to the coast in the summer and fall (Bowman, 1971). However, Sutcliffe (1950) indicated that the species was present only on occasion in the Beaufort Inlet. The results of the laboratory rearing studies reflect these seasonal patterns. Since diapause eggs were not consistently obtained under short day length conditions in the laboratory it seems likely that only a small portion of the females occurring in the Beaufort region have the genetic capacity to produce diapause eggs. Thus if the animals used to start an experiment had the genetic capacity to produce diapause eggs, then such eggs would be produced by their offspring under the expected short day length regimens in the laboratory. However, if the animals lacked the genetic capacity then regardless of the environmental conditions imposed in the laboratory only subitaneous eggs would be produced. If the frequency of individuals possessing the genetic capacity to produce diapause eggs is small, this would account for the inconsistent results obtained in the laboratory. The significant variation that is evident among the cultures reared at 14.5°C, 18L–6D may be due to the fact that carboys B6 and B7 were third generation offspring, but B8 was a first generation culture. However, a similar comparison between F3 (first generation) and F4 (third generation) from Ft. Pierce at 14.5°C, 12L–12D is not significant. There were no difficulties with the incubator conditions or with the algal cultures used in the diet when these experiments were conducted.

The occurrence of diapause egg production in the populations from the Carolinian Province contrasts with that observed for populations in the Virginian Province from Delaware Bay (this study) and from Vineyard Sound (Marcus, 1980a, 1982b). The particular response of the population from Delaware Bay reflects the need for a diapause stage to cope with the harshness of winter as is the case for the animals that occur in Vineyard Sound. However, in the more southern locality (*i.e.,* Delaware Bay) favorable conditions for growth, development, and reproduction persist for a great portion of the year. In this region planktonic stages are present from May to January (Deevey, 1960; Cronin *et al.,* 1962). Since *L. aestiva* were collected from Delaware Bay only during the month of July in this study, it is not known at what time during the fall diapause egg production begins. As expected, diapause eggs were not obtained from females collected in July. To take advantage of the longer growing season, it is likely that diapause egg production does not begin in September as in Vineyard Sound, but rather is delayed by one to two months. From October to November in the Delaware Bay region the period of day length ranges from about 11.75 to 10.5 hours (U. S. Dept. of Commerce, 1983). Thus photoperiodic cycles somewhere in this range should result in diapause egg production by Delaware Bay animals, but not a 12L–12D cycle. These predictions are supported by the laboratory results as evidenced by the still high levels of subitaneous egg production by Delaware Bay animals at 12L–12D, at 18°C. Only with the intermediate photoperiodic cycle of 10L–14D was diapause egg production markedly increased.

The diapause responses expressed by the laboratory-reared and field populations of *L. aestiva* from the different latitudes are similar to those of many insect species. For these animals as for *L. aestiva* the critical day length (*i.e.,* that which induces greater than 50% diapause) tends to be longer in northern populations than in southern populations. The divergence among insect species is most for those species which have a limited capacity for dispersal and migration (see Danilevsky, 1965; Dingle, 1978; Beck, 1980; Denno and Dingle, 1981; Dingle and Hegmann, 1982). Thus, for *L. aestiva,* either such dispersal does not exist or selection overrides the homogenizing effects of any gene flow that may occur. The females that occur in Vineyard Sound, but do not produce diapause eggs (Marcus, 1982a) may be indicative of genetic input from southern regions (*e.g.,* Ft. Pierce) via the hybridization of individuals. That such mixing may occur in the field is suggested by the successful interbreeding of copepods from Vineyard Sound and Ft. Pierce in the laboratory (Marcus, unpub.). Such mixing in the field should promote variability within the populations as suggested by Marcus (1982a).

It is commonly held that the formation of new species reflects the accumulation of genetic differences (Mayr, 1963). But how much or what kind of differentiation is necessary to achieve this distinction is not understood (see Milkman, 1982). It is assumed that genetic adaptation to spatial variation and isolation by distance are of primary importance to the process, but an additional factor which may be important is adaptation to temporal variation (Mayr, 1963; Tauber and Tauber, 1977). In his paper on the paradox of the plankton, Hutchinson (1961) suggests that the rich species diversity of planktonic communities in a seemingly homogeneous realm could be attributed to temporal heterogeneity. In other words, by partitioning the environment on a temporal scale, differences in the timing of occurrence of individuals may reduce competition and enable more species to exist in a given area. Although we no longer regard the marine planktonic system as spatially homogeneous, temporal heterogeneity provides an additional dimension for divergence to occur. If so, then the existence of mechanisms that synchronize the timing of life cycle phases with the environmental regime must be crucial to the survival of species. Some of these mechanisms may be life history adaptations that ensure the accurate coordination of development, growth, and reproduction with favorable periods in the environment and conversely the coupling of periods of dormancy, migration, and dispersal with unfavorable periods. Danilevsky (1965) and Tauber and Tauber (1981) rank diapause as the most important factor contributing to the synchronization of insect life cycles, and this may well be true for many marine planktonic organisms. The diapause response may lead to the reproductive isolation of populations if gene flow is reduced or prevented due to their separation on a temporal scale. Thus the timing of the appearance and disappearance of life history phases resulting from diapause may be of fundamental importance in the speciation process.

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TRANSLOCATIVE FUNCTIONS OF THE ENIGMATIC ORGANS OF STARFISH—THE AXIAL ORGAN, HEMAL VESSELS, TIEDEMANN'S BODIES, AND RECTAL CAECA: AN AUTORADIOGRAPHIC STUDY

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ABSTRACT

Starfish (*Echinaster graminicolus*) were fed C^{14}-labeled clams or liquid glucose-amino acid medium, and subsequently examined as autoradiographic sections. Ingested tracer was first incorporated into the cardiac stomach and lower-middle digestive gland. By 8 to 12 hours, increasing amounts were located throughout the axial organ, aboral hemal ring, radial hemal strand, and mesenteric hemal vessel. Subsequently (22 to 48 hours), label progressed into the genital hemal connectives and the gonads, as well as the connective tissue-hemal plexus of the tube feet. Clearly, hemal tissues, together with associated perihemal spaces, play major roles in the translocation of nutritive materials to the gonads and parts of the tube feet. These nutritive materials, however, may not come directly from the digestive system, which only has poorly developed hemal connections, but from coelomic sources.

Also noted was a gradual build-up of labeled material in the rectal caeca, suggesting that these organs remove nutrients from circulating digestive fluid before its evacuation through the anus or mouth. In animals fed liquid medium, tracer rapidly appeared in all parts of the water vascular system, showing that inflow of sea water does occur through the madreporite. Intense, rapid uptake by the Tiedemann's bodies revealed that they must filter a portion of this inflow, possibly producing coelomic fluid for body turgor and stomach inflation.

INTRODUCTION

The so-called "hemal system" of echinoderms has been the subject of much speculation but little experimental study (see review of Ferguson, 1982b). In starfish, this system consists of a peculiar "axial organ" lying in a coelomic space (axial sinus) adjacent to the stone canal, connecting strands (not really vessels) of "hemal" tissue located in the perihemal coelomic space above the radial nerve cord, and diminuitive aboral continuations to the several digestive organs and the gonads. Histologically, the system does not possess the normal characteristics of a circulatory system, and it appears to be unnecessary for that role since translocations of respiratory gases and nutrients are carried on by the coelomic fluids (Budington, 1942; Ferguson, 1964a, b).

Unexpected evidence that the hemal system does have a role in nutrient translocation, however, came forth in Ferguson's (1970) study on the incorporation of injected labeled amino acids by a starfish. Tracer accumulated in the radial hemal strand and in the connective tissue-hemal plexus of the tube feet. Unfortunately, this study did not examine other parts of the body, and thus, could only indicate a local translocative role between hemal tissue and the tube feet. More recent work, notably

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that of Broertjes *et al.* (1979, 1980), also suggests an active transport role for the starfish hemal system, but the details are as yet unclear.

If the starfish hemal system has significant translocative functions, it should be possible to demonstrate movement of ingested nutrients into and through it. The present investigation was designed to do exactly that—to delineate autoradiographically the distribution of labeled food material in starfish tissues at various times after ingestion. Serendipitously, the study has shed light on not only the translocative role of the hemal organs, but also on the functions of several other enigmatic structures; most notably the rectal caeca, additional parts of the digestive system, the Tiedemann's bodies, the stone canal, and other components of the water vascular system.

MATERIALS AND METHODS

Starfish were taken from the same population of Tampa Bay *Echinaster sp.* used by the author in numerous previous studies. These animals have recently been renamed *Echinaster graminicolus* (Campbell and Turner, 1984). They were obtained in early June, just after the spawning period, and were kept in the laboratory for two days before the start of the experiments. Two different methods were employed to induce them to ingest labeled nutrients whose subsequent distribution in the bodies could be followed.

The first set of experiments employed the recently confirmed (Ferguson, 1982a) ability of small bivalves (*Donax*) to rapidly take up dissolved nutrients from sea water. A group of these clams were placed for 12 hours in a dish containing 100 μCi C^{14}-labeled amino acids (synthetic algal protein hydrolysate) in filtered sea water. They were then thoroughly rinsed, broken open, and fed individually to starfish in a shallow container flushed with a strong stream of fresh sea water (to carry away any released dissolved label). Within 20 minutes all the animals had assumed a feeding posture, which they retained until removed or until digestion was completed 8 to 12 hours later. Individual starfish were removed at timed intervals (2, 4, 6, 8, 12, 24, and 48 hours after the beginning of feeding), immediately placed in ice cold (to slow decalcification) Bouin's fixative, and partially dissected. These animals were subsequently referred to as the "solid food group."

The second series of experiments used the observation (Ferguson, 1969) that *Echinaster* can be induced to directly ingest nutrient-rich sea water. A mixture of 375 mg glycine, 990 mg glucose, 100 μCi C^{14}-labeled amino acid mixture, and 10 mg chloramphenicol was dissolved in 1 l of filtered sea water. A group of starfish was then placed in this medium, and most of the animals soon were seen with everted stomachs in distinctive feeding postures. Specimens were removed, rinsed, and fixed in cold Bouin's after 3, 4.5, 6, 8, and 12 hours in the medium. The remaining animals were then placed in a large container of sea water before sacrifice at 22 and 31 hours from when they were first placed in the medium. It was hoped that this second series would produce much higher specific activities of label in the tissues, although uptake of label by the external parts would have to be allowed for in data analysis. These animals were subsequently refered to as the "liquid medium group."

The specimens from both groups were permitted to slowly decalcify in several changes of cold (4°C) acidic fixative over the next 7–10 days. They were then brought up to and repeatedly extracted in 70% ethanol to remove picric acid. Eventually, they were dissected into disc and arm pieces and prepared as 10 μ-thick paraffin sections. About 40 slices of serial transverse sections, taken from the bivial interbrachium to the mouth, were prepared from each animal, together with several additional slides of arm sections. These were examined, still paraffinized, and 10 to 15 containing representative regions were selected for further study. They were then deparaffinized, wrapped in Kodak AR-10 stripping film, and kept together with dessicant in dark boxes in a refrigerator until developed, 5 to 12 months later. The final autoradiographs were left unstained, and were mounted under glycerine jelly for examination.

These autoradiographic methods permit the visual localization to within a few microns of deposited, insoluble (usually proteinoid) material derived from the original labeled amino acids. Any tracer that was metabolized and excreted was, of course, lost. Thus, while positive exposure of the photosensitive emulsion indicates the definitive presence at that site of derived nutritive material, other, unexposed areas may have contained varying amounts of unregistered label in the living animal. These considerations must be carefully weighed in interpreting the results, and particular care must be exercised in quantitative judgments.

RESULTS

Distinctive morphology

The internal structure of *Echinaster graminicolus* has been partially described in earlier reports (Ferguson, 1969, 1970) and is similar to that of related species (see Cuénot, 1887; Anderson, 1960). Distinctive is its small, eversible cardiac stomach, which connects to duct structures consisting of a series of tangential, parallel ciliated channels (Tiedemann's pouch or diverticulum) lying beneath the lower proximal part of the paired digestive glands of each arm. Aborally, each digestive gland merges into a poorly defined pyloric stomach, which, in turn, connects to a short intestine to which is attached a conspicuous pair of rectal caeca.

Photographs of autoradiographic sections of these and other parts are provided in Figures 1 to 24, arranged in time sequence. They show many newly observed anatomical details, especially of hemal tissue distribution. Clearly revealed are the small irregular, mesenteric hemal "vessels" found at the junctures of the digestive glands and their supportive paired mesenteries (Fig. 12). In the disc, these join together with other very small vessels on the stomach supportive ligaments and an aboral hemal ring embedded in the integument (Figs. 7, 23). Separate "gastric hemal tufts," reported in other species, apparently do not occur in *Echinaster.* The aboral hemal ring connects conspicously (Figs. 7, 9, 13, 14, 23) to the axial organ lying adjacent to the stone canal (Fig. 13 and others). The axial organ is composed of a histologically complex series of spongey sinuses. It is entirely contained in its own chamber (the axial sinus), which is an extension of the perihemal coelomic compartment. The wall of this sinus is rather thick in the middle (Fig. 17), but much thinner at its upper and lower ends (Figs. 9, 18, 23). No distinct connections could be seen between it and the perivisceral coelom or the water vascular vessels. Aborally, the axial organ terminates in a stubby "head" process (Figs. 9, 23), and orally it connects to the oral hemal ring (Figs. 2, 19) surrounding the mouth. From the aboral hemal ring also radiate, within perihemal channels contained in the body wall, genital hemal "connectives" to the genital hemal sinus of each gonad (Figs. 15, 16, 24). The oral hemal ring connects to five ambulacral radial hemal strands, which give off branches to a connective tissue-hemal plexus located in each tube foot (Fig. 11).

The photographs also show other characteristic features of the water vascular system. *Echinaster* has an aboral madreporite which opens into a ciliated stone canal containing a central "T"-shaped ridge (Figs. 5, 6, and others). Orally, the stone canal connects to a ring canal which medially gives off a number of spongey Tiedemann's bodies protruding into the perivisceral coelom (Figs. 1, 2, 19). From the ring canal also extend the radial canals in the ambulacrum of each arm, which give off valvulated transverse vessels to the ampullae and tube feet (Figs. 11, 20). Beneath the irregular coelomic lining of the tube feet is a muscle layer, connective tissue-hemal plexus, and thick columnar epidermis (Fig. 11). The epidermis is highly rugose, especially at the mid level of the tube foot (Fig. 20).

Tracer distribution, solid food group

Regions of retained radioactivity were detected in the internal parts of all the specimens fed solid food, but the levels were generally low, and assessment had to be accomplished primarily by comparing grain densities of selected areas. Table I summarizes the overall interpretation of many individual observations of different sections and regions. While useful comparative differences are implied by the number of plus symbols, a truly quantitative evaluation did not seem justified. In addition to the structures shown, label was detected in irregular patches of the lining of the cardiac stomach, just inside the buccal membrane, and in the oral regions. Relatively little activity was found in the pyloric stomach or the rectal caeca, except after 12 or 24 hours.

The table shows the progressive increases in incorporation of label seen in the digestive glands throughout the 12-hour feeding period. This activity was largely localized in the mid to lower regions. Notable, but lower levels of activity were found in the Tiedemann's diverticula, mainly near the adhesive zones. The label was retained primarily in the inner third or half of the columnar lining cells of the digestive glands, somewhat inward of the lumenal border. In specimens sacrificed 24 and 48 hours after beginning feeding, the distribution was more uniform (and not as locally intense), but similar in basic pattern. These latter animals possessed a distinct zone of labeling located in the connective tissue-hemal plexus on the coelomic side of the digestive organs.

Table I also shows a progressive appearance of label in the major hemal structures. Particularly noteworthy was an observed build up by 12 hours of radioactive substance in the axial organ, and later appearances in the genital hemal connectives and genital hemal sinus.

TABLE I

Relative intensity of label in Echinaster *digestive and hemal tissues after ingestion of solid food*

Hours from start	2	4	6	8	12	24	48
Tiedemann's diverticulum	0	0	++	+	+	+	+
Mid digestive gland	0	++	+++	++++	++++	+++	++
Upper digestive gland	0	+	+	+	++	++	+
Mesenteric hemal vessel	0	0	0	++	++	+++	+++
Aboral hemal ring			0		++	++++	+++
Upper axial organ			0		++	+++	++
Lower axial organ			0		+	++	+
Genital hemal connective			0		+	0	+++
Genital hemal sinus			0		0	0	+++
Oral hemal ring			0		0	+	++
Radial hemal strand	0	0	0	0	++	+	++
Tube foot hemal plexus	0	0	0	0	+	+	+

TABLE II

Relative intensity of label in Echinaster *digestive and hemal tissues after ingestion of liquid medium*

Hours from start	3	4.5	6	8	22	31
Cardiac stomach		+++	++++	++++	+++	+++
Pyloric stomach		0	0	0	+	+
Rectal caeca	0	0	0	+	++	+++
Tiedemann diverticulum	++	+++	++	++	+++	+++
Mid digestive gland	+	++	+	+++	+++	+++
Upper digestive gland	0	+	0	+	++	++
Dig. gland hemal plexus	0	+	0	++	++++	+++
Mesenteric hemal vessel	0	0	0	+	++++	++++
Aboral hemal ring	0	0	0	++	++++	++++
Upper axial organ	0	0	+	++	++++	+++
Axial organ head process	0		0	+	+	++
Lower axial organ	0	0	+	++	++++	+++
Genital hemal connective	0	0	0	+	+++	++++
Genital hemal sinus	0	0	0	+	++++	++
Oral hemal ring		+		+	+++	++++
Radial hemal strand	0	0	0	+	+++	++++
Tube foot hemal plexus	0	0	0	0	++++	++++

Tracer distribution, liquid medium group

The animals fed liquid medium, as expected, ingested a much higher specific activity of nutrients than those of the solid food group. This permitted a more accurate visual localization of tracer in their tissues by noting relative darkening without, in most cases, resorting to counting individual grains. Table II summarizes the observed progressive appearance of label in the digestive, hemal, and reproductive systems. The Figures (1 to 24) represent examples of specific autoradiographs, arranged to show the progressive changes that occurred. These figures illustrate many details of label distribution and variability beyond those evident in the tabulated results.

Before proceeding, it first must be noted that in all specimens a large quantity of tracer must have directly entered the madreporite and become incorporated throughout the lining of the water vascular system, and in amoebocytes found in this system. Most notable was the uptake on the lateral margins of the inner ridge of the stone canal (Figs. 6, 13, and others), the lining of the ring and radial canals (Figs. 2, 11, 20), and the Tiedemann's bodies (Figs. 1, 2, 19). Lesser amounts were seen in the coelomic linings of the tube feet and ampullae (Figs. 11, 20). Labeled material did not appear to have moved through the linings of these structures into other tissues.

The major quantity of tracer taken up by the animals was, of course, ingested. After only a few hours, some label had been incorporated into the tissues of the cardiac stomach, Tiedemann's diverticula, and mid to lower portions of the digestive glands. Within the Tiedemann's diverticula, this label was initially localized near the adhesive zones which form the parallel tangential vessels (Fig. 3), and in individual cells, mainly in the upper lateral portion of the lower duct (Figs. 1, 3).

By 8 hours, the distribution of tracer in the digestive glands was a little more complete, but with concentrations still in the lower portions of the festoons, and near the base of the columnar cells (Figs. 4, 7). The adhesive zones of the Tiedemann's diverticula continued to retain the most activity, except for the immediate junctures (Fig. 4). In later animals, there were increased accumulations of radioactive material in the mid and upper digestive gland, more uniformly distributed throughout the cells (compare Figs. 7, 10, 12, 23). Another major change was the gradual build up of evenly distributed label in the rectal caeca. By 31 hours this build up was quite marked (Fig. 21).

Prominent levels of tracer first appeared in the hemal tissues after 6 to 8 hours. At that time it was found simultaneously in the mesenteric hemal vessels, aboral hemal ring, and fairly uniformly throughout all portions of the axial organ except its very upper and lower ends (Figs. 6, 7, 9). Lesser amounts were found in the other hemal tissues, including the digestive gland hemal plexus, genital hemal connectives (Fig. 8), genital hemal sinus, oral hemal ring, and the radial hemal strands. By 22 and 31 hours, almost all these hemal areas had become very intense with label, which had also spread into the hemal plexus of the tube feet (Fig. 11), and strongly into the genital hemal sinus (Figs. 15, 16). While the hemal layers of the digestive glands showed distinct concentrations (Figs. 12, 22), as did the mesenteric hemal vessels, connections between these were rare and intermittent (Fig. 12). Some activity could be traced nearly continuously from the mesenteric hemal vessels to the aboral hemal ring, to the axial organ, and to the genital hemal connectives (Fig. 23). Much of this appeared to be contained in the fixed hemal substance rather than in cellular components.

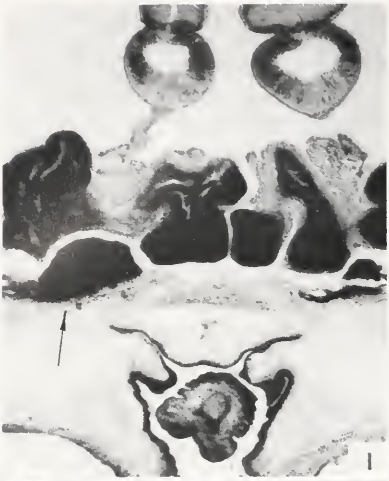
As shown in Table II, the build up of activity in the genital hemal sinus, lower genital hemal connectives, and the connective tissue-hemal plexus of the tube feet occurred after that of the axial organ and the aboral hemal ring. Evidence for progressive build up of activity (as by flow of hemal substance) from the mesenteric hemal vessels of the digestive glands to the aboral hemal ring and the axial organ was looked for but not found; comparison of successive sections showed that the activity appears in most areas of these structures gradually and nearly simultaneously. Cells on the walls of the sinus-like chambers which contain the hemal tissues became somewhat irregularly labeled, and seemed to increase in radioactivity in a pattern similar to the hemal structures themselves (Figs. 11, 14, 15, 18). Of course, movements of dissolved labeled products by the fluids contained within these chambers were not observed directly by the methods employed.

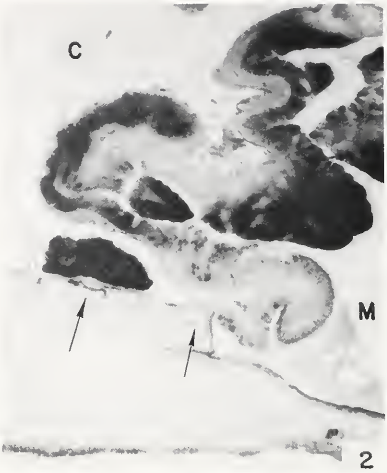
Finally, it may be noted that one animal, sacrificed at 12 hours, apparently failed to ingest significant amounts of the tracer. Like other specimens, radioactivity was found in its water vascular vessels and Tiedemann's bodies, although at a somewhat lower level (perhaps reflective of more quiesent behavior during the feeding period). Of particular interest is the fact that only background levels could be detected in its axial organ and hemal tissues. Hyman (1955) and others have described openings between the ampullary chamber of the stone canal and the axial sinus. In the present material, no such openings were found and the levels of labeling in the upper ends of the axial organs were less than elsewhere in the organs.

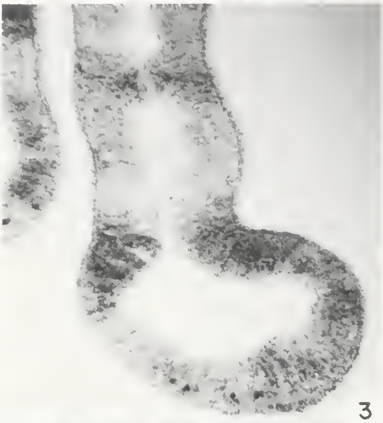
DISCUSSION

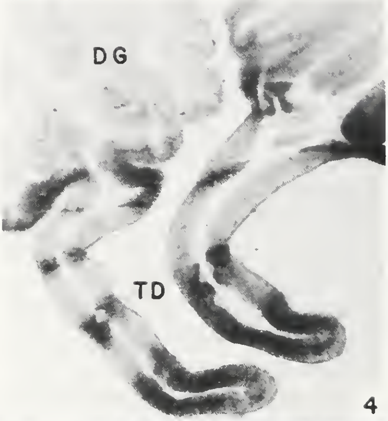
Hemal system function

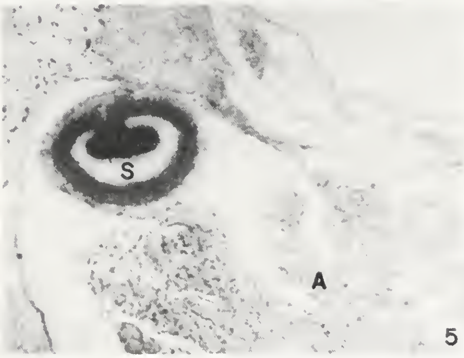
Demonstration of circulatory-type functions in echinoderm hemal systems by using markers has been previously attempted by a number of workers, including Cuénot (1901), Oomen (1926), Stott (1955, 1957), Campbell (1966), Rosati (1970), and Broertjes *et al.* (1980). Since these investigators mainly relied on non-nutritive tracers, their results left many unanswered questions. Ferguson (1963a, b), tried to overcome this problem by feeding small clams injected with C^{14}-labeled nutrients to *Asterias forbesi,* and following the tracer's distribution with autoradiographic and counting methods. Activity was found retained principally in the digestive glands,











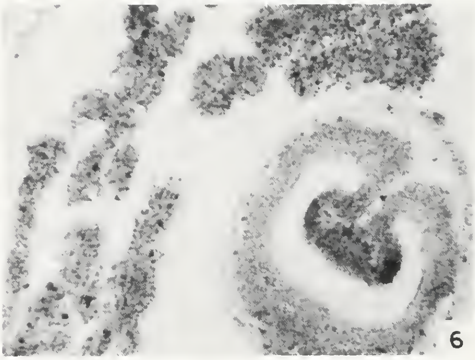


FIGURE 1. Like other figures, an unstained autoradiograph of a section through the oral disc of *Echinaster* fed labeled liquid food. Sacrificed 4.5 hours after start. Arrow shows intense activity in a Tiedemann's body, which connects to the ring canal on left. Above it are heavily labeled portions of the cardiac stomach. Above these are the lower parts of the Tiedemann's diverticula. 80×.

FIGURE 2. Near mouth region after 8 hours from start. Left arrow points to intense label in ring canal and Tiedemann's body; right arrow to weakly labeled oral hemal ring, located in perihemal sinus above nerve ring. C = coelom; M = mouth. Above mouth is cardiac stomach. 80×.

although erratic patches were noted in the hemal septum and other tissues. In light of the dominance of coelomic translocative mechanisms, the importance of the hemal system was discounted. In later experiments, however, stronger evidence was found that the hemal system was involved in nutrient translocation. Labeled amino acid injected into the body cavity of *Echinaster* was located intermittently in parts of the radial hemal strand and the connective tissue-hemal plexus of the tube feet.

The present study provides a much more complete picture of nutrient translocation through the hemal system. It demonstrates that within a few hours of their ingestion, labeled nutrients preferentially begin to appear in the axial organ, and at a somewhat slower rate, in the ambulacral radial hemal strands and other hemal structures. Retention is primarily in the form of non-cellular hemal substance, which after fixation is insoluble in the normal histological solvents. The origin of this material is still unknown, but its nearly simultaneous appearance in multiple locations suggests that it may be initially dispersed through the coelom.

Labeled hemal material appears to move from the radial hemal strand to the connective tissue-hemal plexus of the tube feet, and on to their distal ends where mucus glands are located (Fig. 11). The question of whether this material supports muscular activity in the tube foot wall could not be answered by the autoradiographic method. It was observed that the thick rugose epidermis of the tube feet have an extraordinary affinity for the labeled liquid medium. This propensity for uptake, coupled with the fact that echinoderms possessing particularly active tube feet have an unusually high metabolic dependence on dissolved exogenous nutrients (Ferguson, 1982c), suggests that tube feet depend heavily on external nutrient sources. The tube foot muscles, however, are located inward of the connective tissue-hemal plexus. While it is possible that nutrients are passed from the epidermis to the hemal tissue, some labeled material was found within the tube feet of animals fed solid food (Table I).

Subsequent to the appearance of label in the axial organ, labeled material was found in the genital hemal connectives and then the genital hemal sinus itself. This is an entirely new observation and it conforms to the hypothesis recently suggested by Walker (1980), that the gonads depend on the hemal system for the collection and storage of nutrients used in gametogensis. It is somewhat surprising that the pattern of movement of labeled material towards the gonads is as obvious as it is considering that only a few weeks earlier the experimental animals had completed spawning. No differences were seen between the males and females.

While it now can be concluded that the starfish hemal system, along with possible other functions, does indeed play a significant role in the distribution of nutritive material within the body, the present study cannot fully explain how the system functions in such translocation. Some additional insights gleaned from the observations, however, may be helpful.

It is possible that nutrients are collected in the hemal layer of the digestive glands, are passed up to the mesenteric hemal vessel, and then pass centrally to junctions

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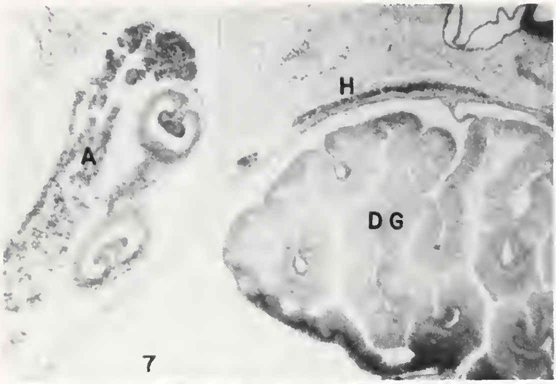
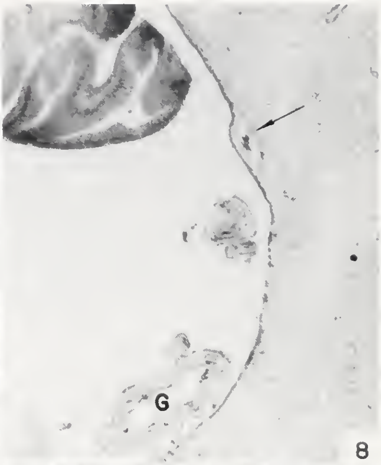
FIGURE 3. Lower Tiedemann's diverticulum, 4.5 hours after start. Maximum label is in cells near adhesive zones and in lower duct. 220×.

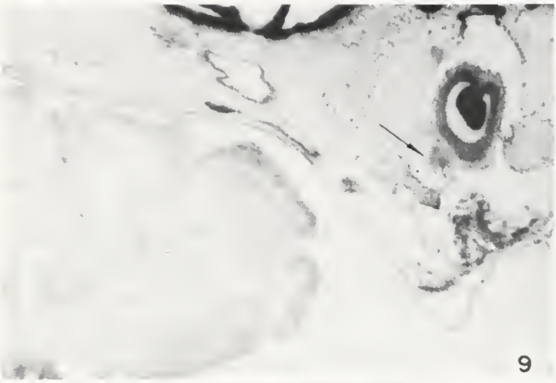
FIGURE 4. Tiedemann's diverticula (TD) attached to digestive glands (DG), 8 hours after start. Maximum activity is in lower duct, adhesive zones, and lower digestive gland. 80×.

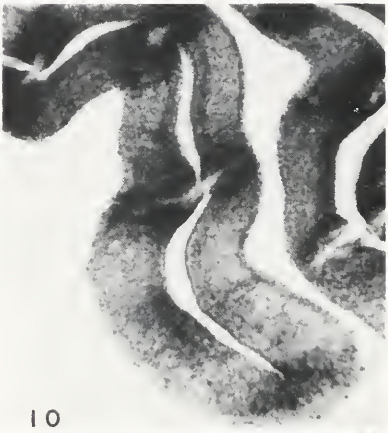
FIGURE 5. Stone canal (S) and axial organ (A), 4.5 hours after start. Intense label is in stone canal, especially on central ridge. Most darkening of axial organ and other tissues is here not label, but natural pigmentation. Aboral hemal ring joins at upper right. 80×.

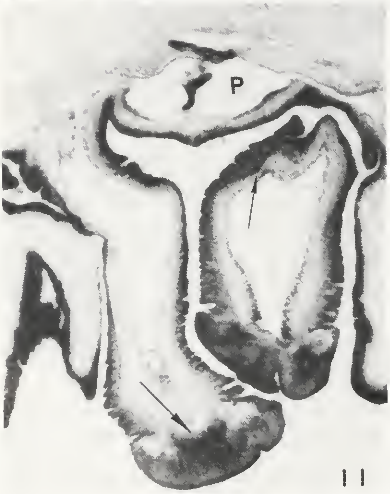
FIGURE 6. Stone canal and axial organ after 8 hours. Most activity is in lateral regions of inner ridge of stone canal. Moderate activity (and pigment) is now located throughout axial organ. 275×.











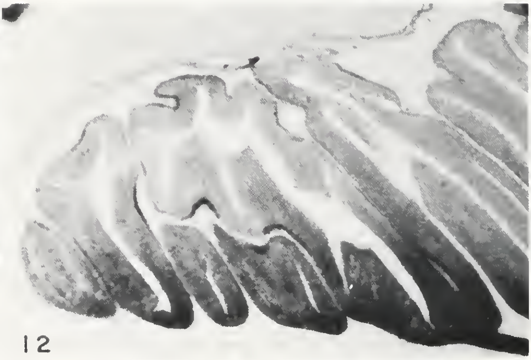


FIGURE 7. After 8 hours. Axial organ (A) and stone canal may be seen within the axial sinus, on left. Aboral hemal ring (H) extends above digestive gland (DG); both it and axial organ contain moderate label. Little label is in upper digestive gland and its supporting mesentery. 80×.

FIGURE 8. After 8 hours. Arrow indicates moderate label in genital hemal connective extending to as yet unlabeled gonad (G). 80×.

FIGURE 9. After 8 hours. A section more central than that of Figure 7. Shows highly labeled upper portion of stone canal near its connection to the madreporite. Arrow points to part of lightly labeled axial organ head process. Edge of the moderately labeled axial organ proper is below it, connecting to aboral

with the aboral hemal ring and the axial organ. Cuénot (1887, 1891, 1948) has described such vessels, and connections between these parts were seen in the present material. Two facts, however, cast doubt on such a interpretation. First, the "vessels" involved are diminuitive and appear incapable of translocating very much material. Indeed, the digestive gland hemal plexus is poorly developed near the mesenteries (Figs. 7, 9, 12). Second, the build up of tracer within these mesenteric vessels and most parts of the axial complex occurs nearly simultaneously. Perhaps a progression was missed between the time intervals selected for study, but that was not the impression gained through careful study of this point with many slides. As noted, a definite progression of label down the genital hemal connectives to the genital hemal sinus was observed.

Earlier workers, dating back to Cuénot (1887, 1901), Chapeaux (1893), and Cohnheim (1901), had developed an alternative hypothesis, that nutrient translocation could be accomplished by migration of amoebocytes. Labeled amoebocytes were searched for in the autoradiographs. Since only a few were seen, mainly in the water vascular system and axial sinus, their significant contribution to the translocative function and the production of hemal substance also can be ruled out.

Broertjes *et al.* (1979), working with *Asterias rubens,* believed that they had found evidence that nutrients were transported centrally in mucus along the inner aboral surface of the digestive glands to the aboral pyloric stomach, and then, by way of the gastric hemal tufts, to the axial organ. In the present study, little tracer was found in the aboral part of the digestive gland or in the pyloric stomach, and structures readily identifiable as gastric hemal tufts did not exist (they may be more characteristic of larger species). The mesenteric hemal vessels appear to join directly with the aboral hemal ring (Fig. 7), with only minor extensions on ligaments extending to the pyloric stomach. While the latter are found to contain varying amounts of tracer, they do not appear to be major conduits.

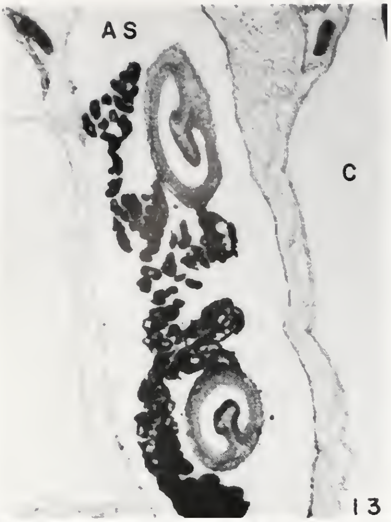
The extensive accumulation of tracer throughout the axial organ, becoming particularly apparent 6 to 12 hours after feeding, suggests that this organ concentrates nutrients reaching it in a fluid state via the coelomic spaces. The digestive glands carry on nutrient exchanges with the circulating fluids of the perivisceral coelom (Ferguson, 1964a, b). While direct connections between the perivisceral coelom and the axial sinus and other perihemal channels could not be found, there are areas where the two compartments are separated only by the thinnest of membranes. These include the upper and lower end of the axial sinus (Figs. 18, 23), and interpodially along the floor of each arm (Ferguson, 1970). Further, the spongey structure of the axial organ (see Bargmann and von Hehn, 1968; Leclerc, 1974) is appropriate for extraction of dissolved metabolites from the surrounding fluid. There was some indication in a few of the sections studied that the distribution of label was more intense

hemal ring extending to left. Note that there is considerably more label in this structure than in the upper digestive gland (left) and its supporting mesentery. 80×.

FIGURE 10. Tiedemann's diverticulum after 22 hours. Maximum label is still near adhesive zones, although distribution is now more general, with some intensification in outer connective tissue-hemal layer. 200×.

FIGURE 11. Ambulacral area of arm near attachment to disc, after 22 hours. Labeled radial water canal is at top, above radial hemal strand, located in perihemal sinus (P), above radial nerve cord. Arrows point to labeled hemal tissues in tube feet; these in other sections connect to the radial hemal strand. Note also, label in nerve tissue bordering the perihemal sinus. 100×.

FIGURE 12. Digestive gland and mesentery after 22 hours. Most label remains in lower part of digestive gland, but very conspicuous amounts have now moved into the connective tissue-hemal layer. One mesenteric hemal vessel is also intensely labeled. 80×.



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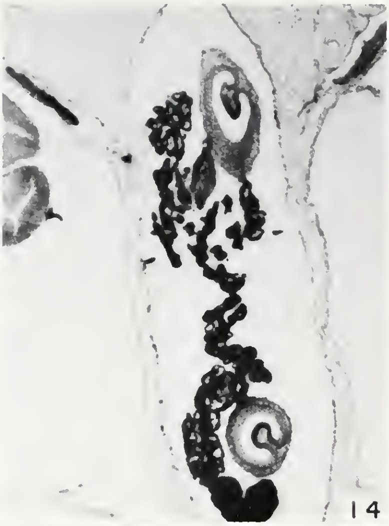

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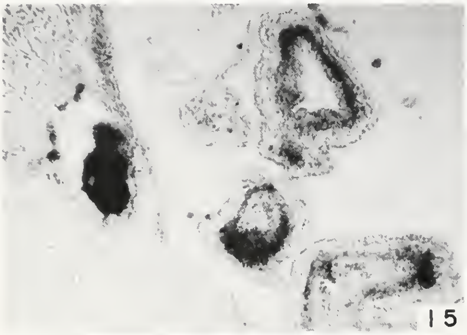

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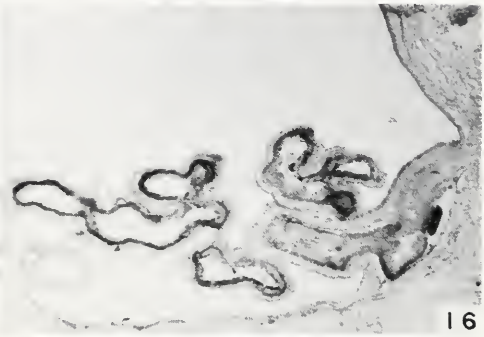

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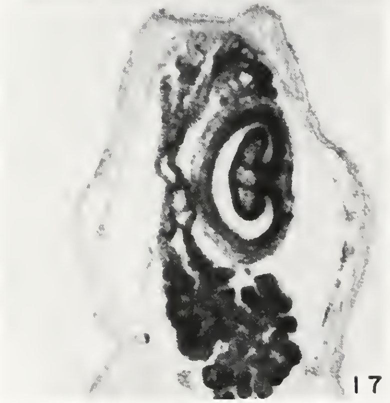

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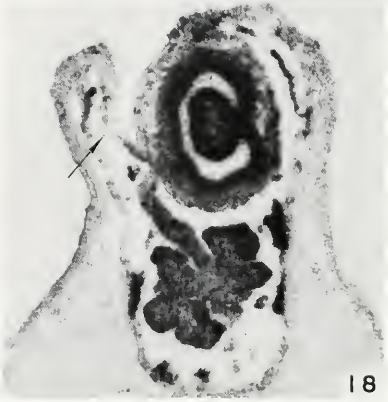

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FIGURES 13 and 14. Sections through two portions of axial sinus (AS) after 22 hours. Heavily labeled axial organ and its upper lateral connecting aboral hemal ring are plainly visible. Lateral walls of axial sinus are thick, separating it from perivisceral coelom (C). 90× and 80×.

FIGURE 15. After 22 hours. Shows intense label now in both genital hemal connective (left) and genital hemal sinus of gonad (center). 220×.

FIGURE 16. Section through another gonad at 22 hours. Genital hemal sinus is very radioactive. Gonoduct extends to right. 90×.

FIGURES 17 and 18. Transverse sections at different levels through axial sinus after 31 hours. Figure 17 is near mid portion; Figure 18 at lower end. Arrow shows thin wall at this point. Note label on inner sinus wall and periphery of axial organ. 135× and 200×.

on the exterior of the axial organ than in the interior (Figs. 17, 18, 23), but this is not certain. Labeled material was seen on the walls of the axial sinus and other perihemal spaces, also suggesting that it was probably present in the fluid.

Even though labeled hemal substances was first seen building up in the axial organ, and then progressively in the genital hemal connectives and genital hemal sinus, the mechanism of transport is uncertain. Detailed study of the hemal tissues shows a structure of congealed material contained in thin-walled, mostly acellular sinuses. While the hemal vessels seem unsuited for axial flow, the adjacent perihemal spaces, by contrast, are lined with flagellated cells oriented to create an efficient local circulation (Walker, 1979, 1980). If label is accumulated within the hemal vessels from the surrounding perihemal fluids, and is unable to flow significant distances, where might it go? An obvious possibility is that much of it could move back into the perihemal fluid for further transport. The hemal material might thus act as a local storage reservoir, while the bulk of the actual transport is accomplished by the perihemal circulation.

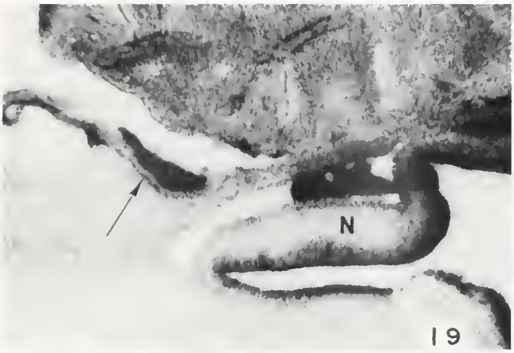
Would there be any functional advantage to such a complex system? This can only be determined through further study, but it is possible. A system of this kind could maintain a low concentration of metabolites in the fluids actually circulating, consistent with the levels in other compartments and osmotic requirements. This low metabolite level, in turn, would be compensated for by the very high concentration contained in the hemal substance. Continuous exchange between the perihemal fluid and the hemal material could produce a net transport. The process may be very roughly analogous to the "facilitated diffusion" mechanism by which myoglobin has been described to enhance oxygen transport in muscle tissue (Wittenberg, 1970). In any case, it is now certain that nutritive metabolites are somehow translocated within the hemal-perihemal complex.

Digestive system

The autoradiographic sections also revealed a number of properties of the digestive system, confirming in some cases conclusions drawn from previous histological studies, and in others, providing new insights. A considerable amount of nutritive material was quickly taken up by the epithelium of the cardiac stomach and the Tiedemann's diverticula (Figs. 1 to 4), showing that the cellular activity of these parts is directly supported by ingested products. The cells located near the adhesive zones and the lower tube of the Tiedemann's diverticum, and in the lower portion of the digestive gland (Figs. 3, 4), must be particularly active. Later, the label in these areas becomes more diffuse, and a new band of intensification develops near the basal connective tissue (Figs. 10, 12, 22, 23).

Interestingly, tracer is slow to move into the upper digestive gland near the mesenteric attachments (Figs. 7, 9). This region is believed to be primarily secretive and cytopoietic as opposed to absorptive (Anderson, 1960; Ferguson, 1966; de Mik-van der Plas, 1981). Only in later specimens (Fig. 23) does a more general distribution of label build up in this location. Since this buildup occurs well after the initial appearance of label in the mesenteric hemal vessels (Fig. 12), these vessels may serve primarily as reservoirs for the nutritional and trophic needs of their adjacent secretory tissues, rather than as conveyors of hemal material to other parts of the body.

The gradual build up of label in the rectal caeca (Table II) is most notable. Possibly these organs serve to remove nutritive material from the digestive contents before they are vented to the exterior. (Fluid and feces are voided by *Echinaster* at frequent intervals.) The described histological structure of the rectal caeca is well suited for such a role (Bouillon and Jangoux, 1970; Jangoux *et al.*, 1975). As there are few



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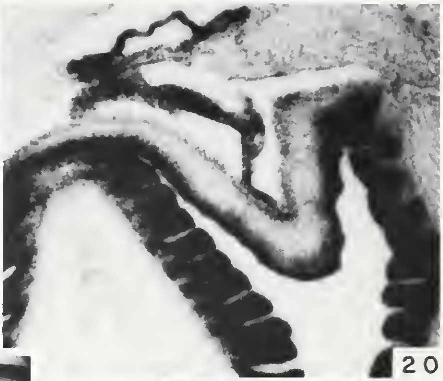

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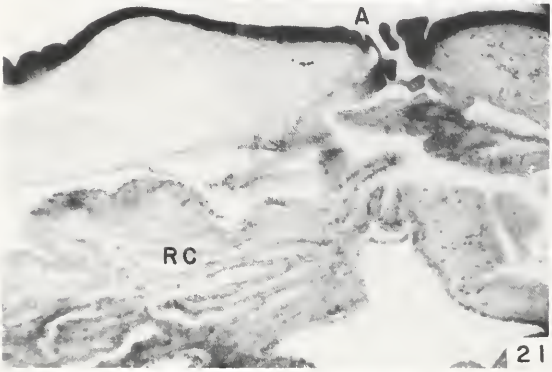

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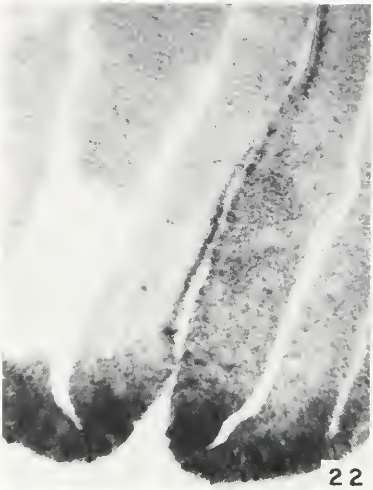

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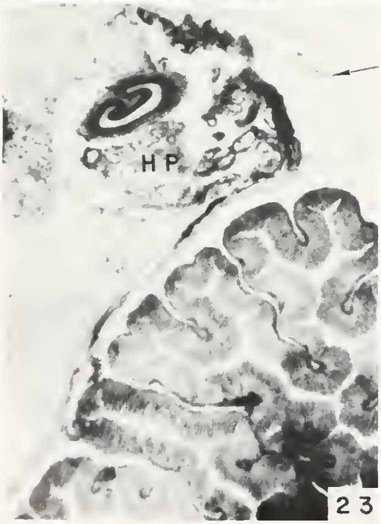

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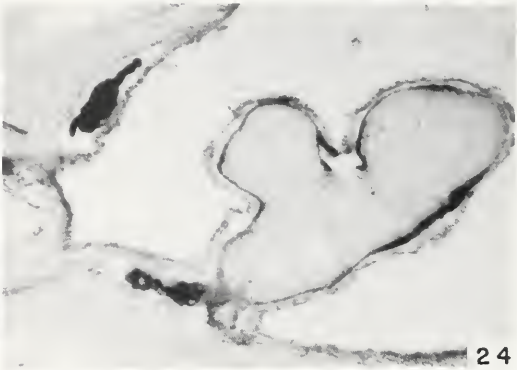

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FIGURE 19. Near mouth after 31 hours. Compare to Figure 2. Arrow points to edge of Tiedemann's body. Hemal ring, above nerve ring (N), is now very heavily labeled. 200×.

FIGURE 20. Ambulacral area after 31 hours. Labeled radial hemal strands may be seen in perihemal sinus above radial nerve cord and beneath radial water canal. Lateral extensions of radial hemal strand connect with connective tissue-hemal layers of tube feet. Rugose epidermis of tube feet and lower radial nerve cord are heavily labeled by direct uptake from exterior. 260×.

FIGURE 21. Rectal caecum (RC) and anus (A) after 31 hours. A moderate level of radioactivity now exists throughout rectal caecum, whereas in earlier specimens it did not. Note that hemal tissues are not conspicuous. Epidermis has taken up label directly from medium. 80×.

attached hemal vessels on these or adjacent structures, most of the scavenged nutrients eventually may be released into the coelomic fluid circulation.

Water vascular system

Except as already noted, label could not be detected in the water vascular system of the solid food group specimens, but was very evident in those of the liquid medium group. In previous studies practically no tracer was seen entering the madreporite nor penetrating to the stone canal or deeper layers of the water vascular system. Ferguson (1967) suggested that this lack of penetration was due to the efficiency of uptake by the outer layers, thereby preventing substances (at normal concentrations) from moving further into the system. An alternative hypothesis had been argued by Binyon (1964, 1966, 1976) and also supported by Prush and Whorisky (1976)—that little water enters the madreporite and that water vascular fluid replacement is accomplished osmotically.

A major difference in the present experiments from previous ones was the large amount of non-radioactive "carrier" used in the nutritive media to induce the feeding reaction. This carrier appears to have "flooded" the transport mechanism of the madreporite epidermal cells permitting the tracer to move rapidly into the deeper vessels. This observation clearly confirms that there is a significant inflow of sea water through the madreporite and into the major parts of the system. The osmotic mechanisms described by Binyon must function secondarily to this inflow.

Also of great interest was the intensive and rapid accumulation of tracer by the Tiedemann's bodies, located on the ring canal (Figs. 1, 2, 19). These organs consist of numerous radiating chambers of flagellated, cuboidal epithelia (Bargmann and Behrens, 1964). Tiedmann (1816) speculated that they filter water from the water vascular system, but later investigators have proposed a number of alternative hypotheses. Hoffmann (1875), particularly, suggested that they function in amoebocyte formation, and this view was further promulgated by Cuénot (1887, 1901) in his earlier work, and others. The absence of elevated cytopoietic activity, however, was demonstrated by Ferguson (1966), who concluded that the organs were specialized for absorption and phagocytosis, and thus served to filter body fluids.

The absorptive properties of the Tiedemann's bodies are very evident in the present work. Sea water taken up through the madreporite must be propelled down through the stone canal and ring canal, and into these structures. As they are made up of blind-ended chambers, a portion of this flow likely passes through them into the perivisceral coelom. Thus, their role now may be seen not only as puritive, but also as probably generative of the perivisceral coelomic fluid itself. This would help to explain the considerable similarity between echinoderm coelomic fluid, water vascular fluid, and sea water (see Binyon, 1966). It would also explain how starfish are able to maintain a turgid composure, especially while protruding and inflating their cardiac stomach during feeding. (Note that the one animal that did not feed had

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FIGURE 22. Digestive gland after 31 hours. While label is more generalized than earlier, much activity is still retained in lower portions. Note intensification in connective tissue-hemal layer. 200×.

FIGURE 23. After 31 hours. Shows upper stone canal, axial organ head process (HP), edge of axial organ, and aboral hemal ring extending above a more generally labeled digestive gland. Arrow points to thin wall of axial sinus in this region. 80×.

FIGURE 24. Genital hemal connective and gonad after 31 hours. Intense label can be seen in the hemal tissues. Gonoduct extends to left. 95×.

lower levels of tracer in its Tiedemann's bodies than did the others.) Certainly, osmotic and other hydrodynamic mechanisms must at times also contribute to fluid replacement, but the importance of the Tiedemann's bodies in this function appears to have been overlooked by modern workers.

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INACTIVATION OF CELL MOVEMENT FOLLOWING SEXUAL CELL RECOGNITION IN *PARAMECIUM CAUDATUM**

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ABSTRACT

A positive correlation between sexual cell recognition and decrease in swimming velocity was studied in the ciliated protozoan, *Paramecium caudatum*. Ciliary membrane vesicles with conjugation-inducing activity rapidly decreased swimming velocity of living cells of the opposite mating type. Immobilization of the ventral cilia and perturbation of metachronal waves in the dorsal cilia were observed upon application of the membrane vesicles. Anterior shift of the beating direction and decrease in the beating angle or amplitude were also observed in the cells reacting to the vesicles. A similar change of swimming behavior and ciliary inactivation was seen in the chemical induction of conjugation. A possible regulatory mechanism of ciliary movement is discussed.

INTRODUCTION

In many ciliates, cilia, the locomotive organelles, also play an important role as a mating organelle. Thus, conjugation in *Paramecium* is initiated by ciliary agglutination between cells of complementary mating types referred to as the mating reaction (Sonneborn, 1937). Cells in the agglutinative mating reaction adhere to each other at the surface of ventrally located cilia (mating reactive cilia) and appear to move slowly or stop swimming for about one hour until conjugating pairs appear. However, the agglutination of the cells hinders detailed observation of the change in ciliary movement of individual cells. Kitamura and Hiwatashi (1980) reported that ciliary membrane vesicles, prepared by treatment of mating reactive detached cilia with lithium diiodosalicylate, have high conjugation-inducing activity but lack the agglutination-inducing activity. The reaction between the membrane vesicles and cells of the opposite mating type provides a good system for detailed observation of ciliary movement during the mating reaction because the reaction involves the same mechanism as the mating reaction of living cells in the induction of conjugation.

Marked change in swimming behavior upon transferring mating reactive cells into conjugation-inducing chemicals has been reported by many investigators (Miyake, 1958; Tsukii and Hiwatashi, 1978; Cronkite, 1979). However, little is known about ciliary movement of the cells in the conjugation-inducing media and also about the precise relationship between the swimming behavior of cells in the mating reaction and its implication in the mechanisms triggering the succeeding mating processes. Here we report detailed observations of ciliary movement following sexual interaction with mating reactive membrane vesicles and treatment with conjugation-inducing chemicals, and also discuss possible mechanisms of the inactivation of cell movement.

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* This paper is dedicated to Dr. Mamoru Kusa on his retirement.

Abbreviations: LIS = lithium diiodosalicylate, Tris = tris(hydroxymethyl)aminomethane, MV = membrane vesicles, EDTA = ethylenediamine tetraacetic acid, ATP = adenosine triphosphate, EGTA = ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

MATERIALS AND METHODS

Stocks and culture method

The stocks used were Kyky 1, Ksy-S7a, and 27aG3-S1 of mating type V, and Yt3-G9, d-8b, and 27aG3 of VI in syngen 3 of *Paramecium caudatum*. Sexually immature cells were obtained from selfing progeny of 27aG3-S1. They are genetically identical to their parent cells because 27aG3-S1 is a complete homozygous clone (Tsukii and Hiwatashi, 1979).

Cells were cultured at 25°C in 1.25% (w/v) lettuce juice medium in Dryl's solution (Dryl, 1959), and inoculated with *Klebsiella pneumoniae* one day before use (Hiwatashi, 1968). Subculture was made frequently to maintain active growth. Stationary phase cells for experiments were obtained by inoculating several hundred cells into 2 ml culture medium, adding fresh medium of 4 ml, 10 ml, and 10 ml on successive days, thus allowing growth to occur to about 2×10^3 cells/ml. At the stationary phase, most sexually mature cells exhibited strong mating activity; immature cells showed no activity. Ca-poor culture medium was prepared to grow cells for chemical induction of conjugation.

Isolation of LIS-membrane vesicles

Ciliary membrane vesicles which can induce conjugating pairs without prior occurrence of mating agglutination were isolated by the method of Kitamura and Hiwatashi (1980). Mating reactive cilia detached by the modified method of Fukushi and Hiwatashi's (Kitamura and Hiwatashi, 1978) were treated with 4 m*M* lithium diiodosalicylate (LIS, Eastman, No 11187) in 10 m*M* Tris-HCl buffer, pH 7.6, for 30 min at 4°C. The concentration of the total ciliary protein in this suspension was about 0.5 mg/ml. The treated cilia were precipitated by centrifugation at $9000 \times g$ for 10 min and the supernate was passed through 0.2 μm Membran-filter (Sartorius SM 11407) to remove possible contamination of remaining cilia fragments. The filtrate was dialyzed against 3 liter of 10 m*M* Tris-HCl, pH 7.3, overnight with a change of the same buffer. The dialysate was centrifuged at $105{,}000 \times g$ for 60 min. The pellet obtained by this procedure always consists chiefly of membrane vesicles which are called the LIS membrane vesicles.

Measurement of swimming velocity

For photographs of swimming behavior, one ml of cell suspensions of 1000/ml to 2000/ml in cell density were applied to glass plates under which water from a temperature-controlled bath was circulating through a thermal stage of glass. Swimming velocity was measured by photographs taken with 2 seconds exposure or stroboscopic pulse exposures. Before measurements, cells were adapted to the solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$ and 1 m*M* Tris-HCl, pH 7.1, for 20 min or longer at 21°C.

Chemical induction of conjugation

Mating reactive cells of a single mating type cultured in the Ca-poor medium were washed twice with and suspended in a solution containing 1 m*M* KCl, 0.03 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1. The cell suspension was mixed in test tubes with the same volume of the inducing medium which contains 19 m*M* KCl, 0.03 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, at 25°C to give final concentrations of 10 m*M* KCl and 0.03 m*M* $CaCl_2$. The cell density after mixing with the inducing medium was about 2000/ml.

RESULTS

Mating type specific inhibition of swimming by the LIS membrane vesicles

The LIS membrane vesicles (LIS-MV) with high conjugation-inducing activity were prepared from about 100 l culture of stock Yt3-G9. When the LIS-MV were added to the opposite type cells, no agglutination occurred but swimming was inhibited within a minute. Selfing pairs were induced in about 50 min if the inhibition of swimming was not disturbed. Neither inhibition of swimming nor induction of selfing pairs was observed when the vesicles were added to cells of the same mating type (Fig. 1). Figure 2 shows the mating-type-specific decrease in swimming velocity upon addition of the LIS-MV. Addition of about 50 μg/ml (protein concentration) LIS-MV decreased swimming velocity of opposite type cells to 50% of the normal velocity in a minute and to 15% in 10 min. The inhibition of swimming was reversible; cells once inhibited returned to the normal swimming speed when they were washed free from the LIS-MV (arrows in Fig. 2B).

Swimming velocity of selfing pairs induced by the LIS-MV was not affected by subsequent addition of the LIS-MV. Selfing pairs were induced by the LIS-MV (50 μg/ml) from cells of opposite mating type. About 7 hours after the addition of the vesicles, induced selfing pairs were washed with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, and added with the LIS-MV (50 μg/ml) of opposite mating type. As seen in Table I, selfing pairs did not decrease their swimming velocity upon addition of the LIS-MV. This result is consistent with the fact that the cells, having formed conjugating pairs, lost their mating reactivity.

Observation of ciliary movement when the LIS-MV were added

When LIS-MV reacted cells were observed under the phase contrast microscope, immobilization of cilia on the ventral surface was seen. Some ventral cilia stuck together at their tips. This observation agreed well with the report by Hiwatashi (1961) that the mating reactivity of *P. caudatum* is restricted to the ventral side of the cell. However, the above observation does not mean that movement of the dorsal cilia is normal. Dorsal cilia did not show typical metachronal waves. An anterior shift of the beating direction and decrease in the beating angle of amplitude were observed. These results suggest that movement of dorsal cilia which lack mating agglutination ability, is also affected by interactions of mating-reactive surface membranes of ventral cilia with the LIS-MV.

Effect of LIS-MV on the swimming velocity of Triton-extracted models

To determine if only living cells show decrease in swimming velocity upon sexual recognition, the LIS-MV were applied to detergent-extracted models of the cells, in which the cell membrane is functionally disrupted.

Triton-extracted models of *Paramecium* were prepared by Naitoh and Kaneko's (1972) method with slight modifications. Cells were suspended in the extraction medium containing 0.005% (v/v) Triton X-100, 20 m*M* KCl, 10 m*M* EDTA, and 10 m*M* Tris-maleate buffer, adjusted to pH 7.0 with NaOH, at 0°C to 1°C. After treatment with the extraction medium for 30 min, the specimens were washed several times to remove the detergent, with a solution of 50 m*M* KCl and 10 m*M* Tris-maleate, pH 7.0. The models were reactivated by adding 4 m*M* ATP, 4 m*M* $MgCl_2$, and 3 m*M* K_3-EGTA to the washing solution 30 min after the washing.

When the models were treated with more than 100 μg/ml LIS-MV (the concentration being enough for the full inhibition of swimming in living cells), no change

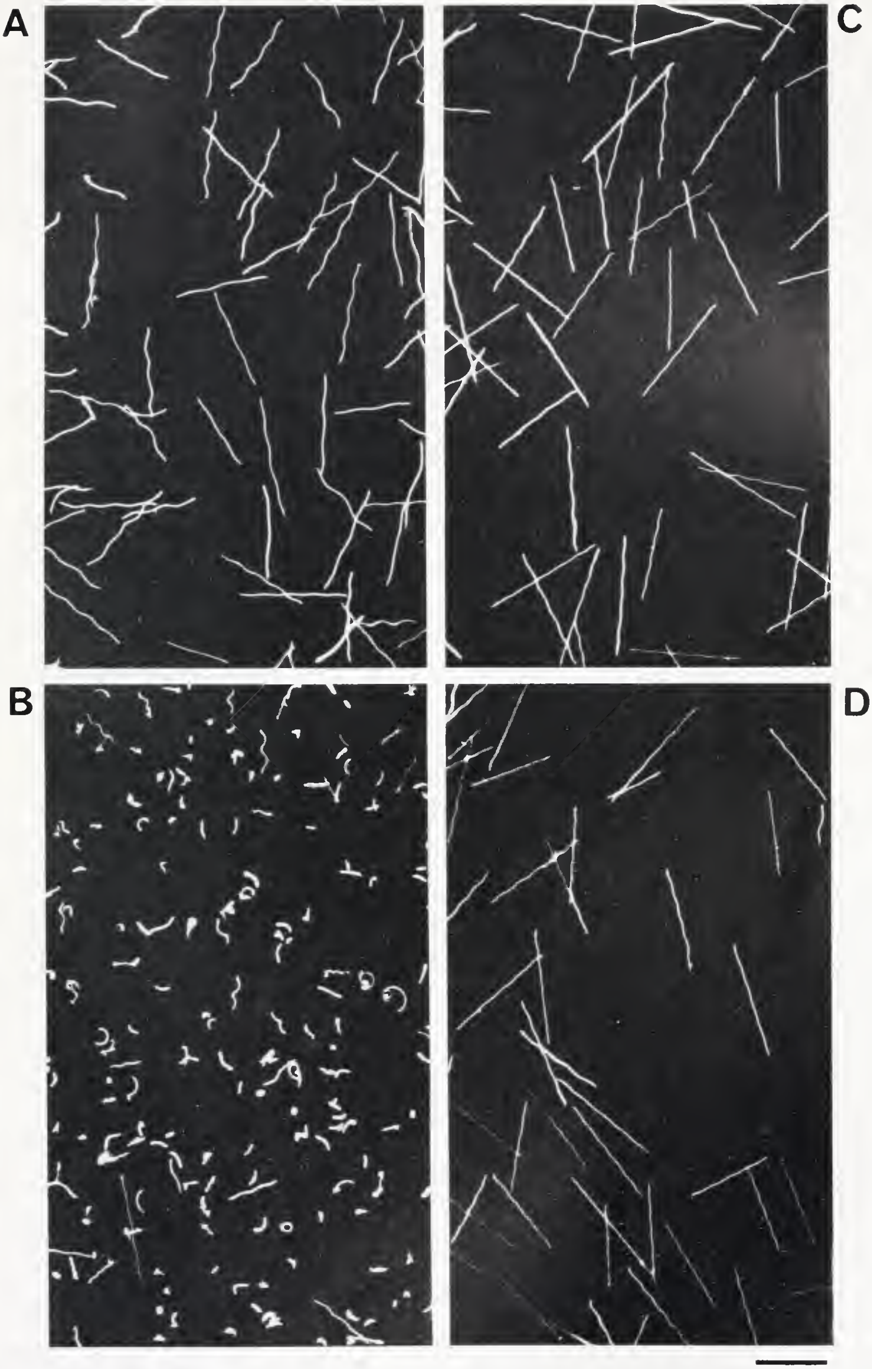

FIGURE 1. Tracks of swimming behavior of *P. caudatum* before and after the addition of the LIS-MV. The photographs were taken under dark-field illumination with two seconds exposure at 21°C. A, C; swimming tracks of cells before addition of the LIS-MV. B, D; swimming tracks of cells 10 min after addition of 40 μg/ml LIS-MV from stock Yt3-G9 (mating type VI). A, B; the opposite mating type cells, stock Kyky 1 (V). C, D; the same mating type cells, stock d-8b as the membrane vesicles added. Bar = 2 mm.

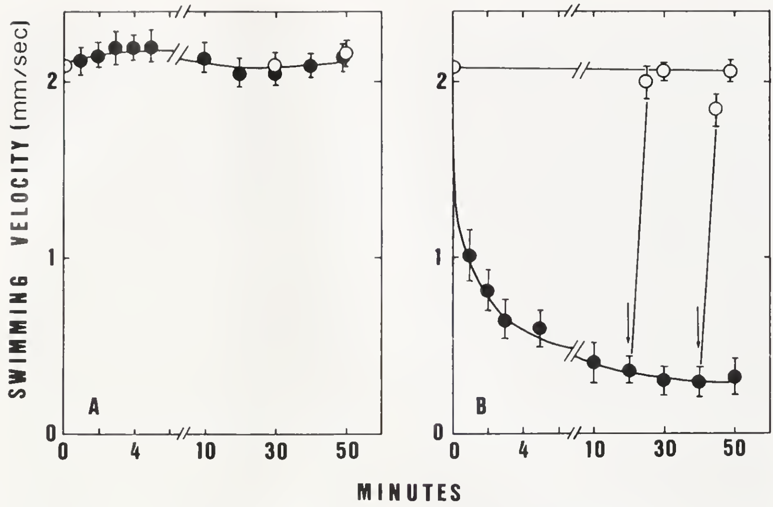

FIGURE 2. Mating type specific decrease in swimming velocity of *P. caudatum* by the LIS-MV. (A) Swimming velocity of the same mating type cells, stock Yt3-G9 as the vesicles added. (B) Swimming velocity of the opposite type cells (stock S7a). The abscissa represents the time in minutes after the addition of the LIS-MV. Ordinate, swimming velocity (mm/s) at 21°C. ●; With 50 μg/ml LIS-MV from stock Yt3-G9, mating type VI. ○; Without the vesicles. Cells were washed to remove the LIS-MV with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, at the times indicated by arrows. Swimming velocity of the washed cells was measured after adaptation for one minute in the solution to settle the cells mechanically agitated by hand-operating centrifugation. Each swimming velocity was determined by about 30 measurements of different tracks.

in the swimming velocity of the models was seen (about 200 μm/s before and after the addition of the LIS-MV). The failure of inducing a decrease in swimming velocity of the models is not caused by failure of the attachment of the LIS-MV to the models. Mating-type specific agglutination of the models was induced by addition of more than 500 μg/ml of the LIS-MV. The results suggest that the inhibition of swimming is not induced by mere attachment of LIS-MV but that some membrane-physiological control of ciliary movement lacking in the Triton-extracted models is responsible for the inhibition of swimming.

TABLE I

*Effect of the LIS-MV on the swimming velocities of single cells and selfing pairs**

	Swimming velocity (μm/s)[a]	
Cells of stock 27aG3	−LIS-MV	+LIS-MV[b]
Single cells	1581 ± 185 (12)	461 ± 148 (12)
Selfing pairs	896 ± 233 (10)	862 ± 68 (9)

* Each result is the mean and 95% confidence limit. Numbers in parentheses indicate number of specimens.

[a] Swimming velocity was determined by measurements of about 10 swimming tracks of different cells by stroboscopic pulse exposures (six pulse exposure at the interval of 0.5 s each).

[b] The LIS-MV from stock Kyky 1 (mating type V).

Swimming behavior during chemical induction of conjugation

In *Paramecium,* various chemical agents under Ca-poor conditions induce selfing pairs among cells of a single mating type (Miyake, 1958, 1968). Selfing pairs appear in about 60 min in the induction medium without prior occurrence of agglutinative mating reaction. Cells show a marked change in their swimming behavior during the chemical treatment (Miyake, 1958; Tsukii and Hiwatashi, 1978; Cronkite, 1979). The change in the swimming behavior of the cells treated with the conjugation-inducing

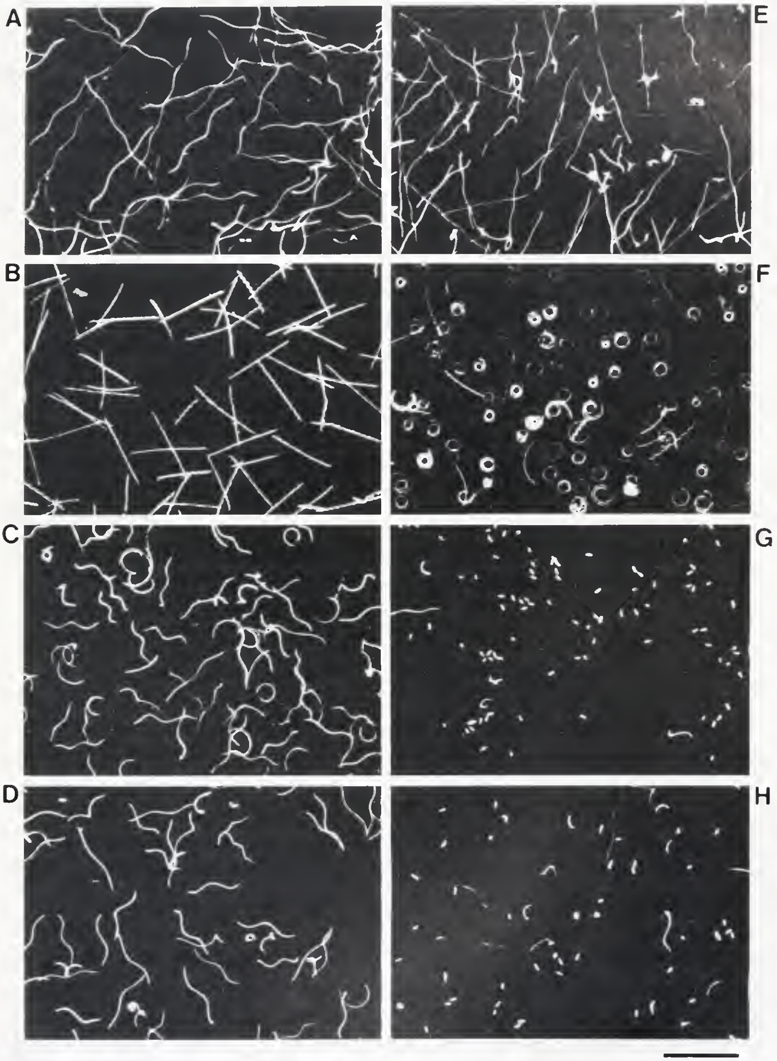

FIGURE 3. Photographs of swimming tracks of *P. caudatum* when introduced into a chemical induction medium. A–D; immature cells of stock 27aG3-S1. E–H; mating reactive mature cells of stock 27aG3-S1. A, E; adapting cells in a solution containing 1 mM KCl, 1 mM $CaCl_2$, and 1 mM Tris-HCl, pH 7.1 at 25°C. B, F; cells in the induction medium 30 s after the transfer. C, G; cells in the induction medium 10 min after the transfer. D, H; cells 40 min after the transfer. The photographs were taken under dark-field illumination with 2 s exposure. Bar = 2 mm.

chemicals consists of cell whirling accompanying a complete stop of forward swimming. For observation of the ciliary movement in the conjugation-inducing medium, we chose a very simple inducing medium containing only KCl and a trace of $CaCl_2$. Effects of these two cations on ciliary movement in *Paramecium* have been well analyzed electrophysiologically. Potassium is one of the best agents for the artificial induction of conjugation (Miyake, 1958).

For observation of swimming behavior of cells in conjugation-inducing chemicals, mating reactive cells were compared with non-reactive cells of the same genotype in the period of sexual immaturity. Thus, mating reactive cells of the complete homozygous strain 27aG3-S1 and immature cells of their selfing progeny were used. Figure 3 shows the change in swimming behavior of mating reactive and non-reactive cells upon treatment with conjugation-inducing chemicals. After being suspended in an adaptation medium containing 1 m*M* KCl, 0.03 m*M* $CaCl_2$, and 1 m*M* Tris-HCl,

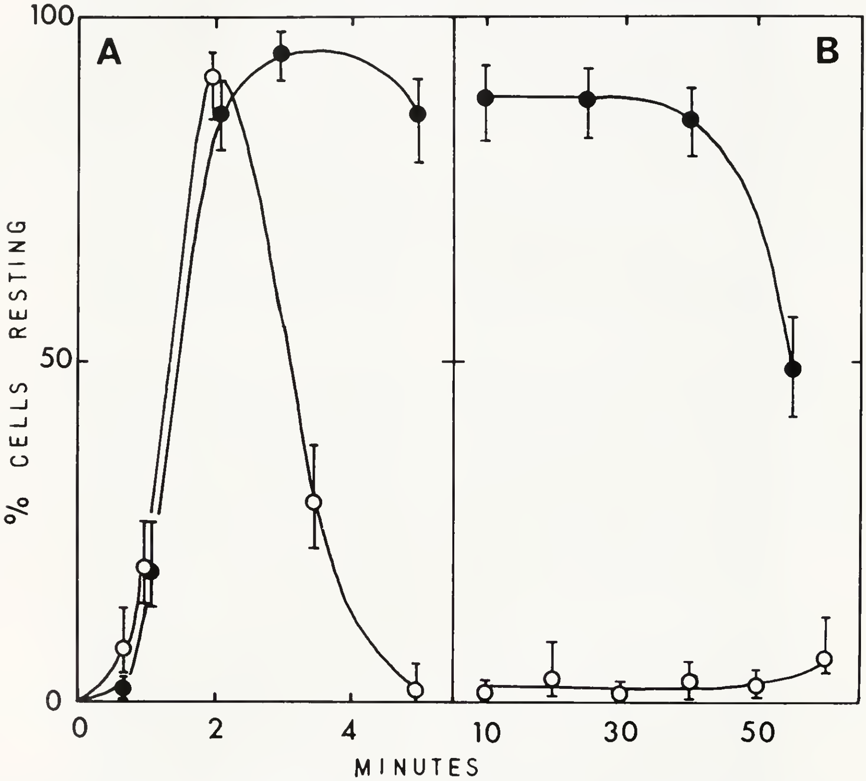

FIGURE 4. Mating reactivity-specific inhibition of swimming in the chemical induction medium. Abscissa; time after the transfer to the chemical induction medium (10 m*M* KCl, 0.03 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1). Ordinate; percent of cells showing cessation of swimming in photographs taken with one second exposure. ○; Cells of stock 27aG3-S1 in sexually immature period. ●; Mating reactive mature cells of stock 27aG3-S1. (A) Time course within 5 min after the induction. Immediately after cells were transferred into the induction medium, one ml of the cell suspension was applied to a glass plate to take photographs at each time. (B) Time course from 10 to 60 min after the induction. One ml of sample cell suspension was applied onto glass plates at each time from the same batch. Photographs were taken one minute after the application of cells to the plates in order to settle the transferred cells from possible mechanical agitation.

pH 7.1, for 10 min, cells were transferred into the conjugation-inducing medium containing 10 m*M* KCl, 0.03 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1 at 25°C.

Immature cells having no mating reactivity showed continued backward swimming for about 40 s. This appears as fine wavy tracks in the photograph (Fig. 3B). In contrast, mating reactive mature cells ceased backward swimming about 20 s after the transfer and showed a whirling motion (Fig. 3F). Another marked difference in swimming behavior between immature and mature cells occurred several minutes after transfer into the induction medium. More than 90% of reactive cells stopped swimming and continued to be in this state until the appearance of selfing pairs (Fig. 3G, H). Neither cessation of swimming nor formation of pairs was induced in immature cells (Fig. 3C, D). Figure 4 shows the percentage of non-motile cells during the chemical induction of conjugation. More than 90% of mating reactive cells showed cessation of swimming within 2 min and remained in this condition for more than 40 min, until the appearance of conjugating pairs. Conjugating pairs swim normally in the conjugation-inducing medium, whereas immature cells did not stop swimming, though their swimming behavior changed considerably in the medium (Fig. 3C). The increase in the number of resting cells in the figure (Fig. 4A) does not indicate the cessation of swimming in immature cells. It indicates whirling cells because cells at the end of backward swimming show small whirling circles. The tracks of such cells cannot be distinguished from those not swimming. Thus, dots in photographs include whirling cells which were scored as resting cells. The immature cells, however, regained their forward swimming shortly after such whirling. When the mating-reactive cells were washed free from the inducing medium with a solution containing 1 m*M* KCl, 1 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1, and resuspended in the same solution, they recovered their forward movement immediately and returned to normal swimming behavior.

Calcium ions inhibit chemical induction of conjugation (Miyake, 1958; Hiwatashi, 1959). When the concentration of $CaCl_2$ was increased ten times that of the normal

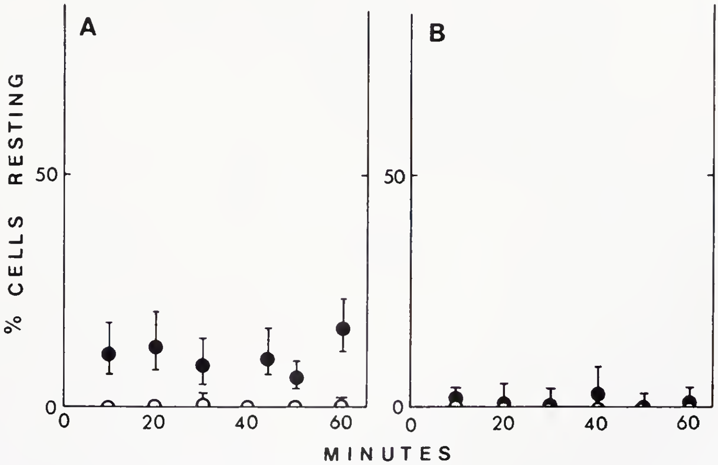

FIGURE 5. Inhibition of the cessation of swimming by Ca ions. (A) Cells in the solution containing 10 m*M* KCl, 0.3 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1. (B) Cells in the solution containing 10 m*M* KCl, 3 m*M* $CaCl_2$, and 1 m*M* Tris-HCl, pH 7.1. Abscissa; time after the transfer to the solution, ordinate; percent of resting cells indicated as in Figure 4. ○; Immature cells of stock 27aG3-S1, ●; Mating reactive mature cells of the same stock.

induction medium, the number of conjugating pairs induced decreased to less than 10%. In this condition a noticeable decrease in the ratio of the resting cells was seen (Fig. 5A). Neither cessation of swimming nor pair formation was observed when the concentration of $CaCl_2$ was 100 times higher than the induction medium (Fig. 5B). These results clearly show a positive correlation between the cessation of swimming and the induction of conjugating pairs.

Observation of ciliary movement during chemical induction of conjugation

When mating reactive cells whose swimming was stopped in the induction medium were observed under a phase contrast microscope, perturbation of ciliary metachronal waves was seen not only in the ventral cilia but also over the dorsal cilia. Moreover, adhesion of some ventral cilia of the same cell at their tips were observed. This is almost the same change observed in cells treated with the LIS-MV.

DISCUSSION

The inhibition of swimming induced by sexual recognition on the ciliary surface in *Paramecium* opens an interesting problem on regulatory mechanisms of ciliary movement. The inhibition appears to couple tightly with the trigger reaction in the conjugation process since conjugating pairs cannot be induced when the inhibition of swimming by the LIS-MV was disturbed by washing cells (Fig. 2B) or when cessation of swimming in the conjugation-inducing medium was inhibited by adding Ca ions (Fig. 5).

Machemer (1974) reported a positive correlation between membrane depolarization and change in ciliary beat frequency or shift of the beat direction. Preliminary results obtained by direct measurement of membrane potential, however, suggest that the ciliary inactivation induced by mating recognition with the LIS-MV is not controlled by the membrane potential change (Kitamura *et al.*, 1980). Furthermore, it takes about 5 min for the cells reacted with the LIS-MV to show complete inhibition of swimming (Fig. 2). If any electrical change in cell surface membrane was responsible for the inhibition of ciliary movement by sexual recognition, the response should be much faster than that obtained. We recently detected an increase in adhesiveness of the ventral cilia to polystyrene surfaces during the mating reaction or the chemical induction of conjugation (Kitamura, unpub. result). This change in the adhesiveness of cells proved to result from an increase in hydrophobicity of surfaces of the ventral cilia (Kitamura, 1982). Thus, ciliary membranes with mating reactivity become more sticky in the conjugation-inducing medium or by the attachment to the LIS-MV and hence agglutinate each other. This change could cause the inactivation of ciliary movement and perturbation of ciliary coordination observed in this study. The result with Triton-extracted models where swimming velocity did not decrease following LIS-MV exposure apparently conflicts with this hypothesis. As described in the results, however, the swimming velocity of the model made from mating reactive cells was about 200 μm/s, while that of the model from non-reactive cells (sexually immature) was about 260 μm/s. This suggests that some inactivation of ciliary movement in the mating reactive models had already occurred in the reactivation medium before adding the LIS-MV, because the reactivation medium itself has a strong conjugation-inducing activity as seen in its ionic condition, high in K^+ concentration and low in Ca^{2+} concentration (50 m*M* KCl and 3 m*M* EGTA). We do not know whether adhesion of ventral cilia occurs in this condition. But, if the adhesion had already occurred, addition of the LIS-MV would not decrease the swimming velocity further.

The observation that the models from mating reactive cells tend to swim in a twisting manner upon reactivation but those from cells without mating reactivity do not, agrees well with this explanation.

An important role of Ca^{2+} has been suggested in the activation-initiating mechanism of conjugation in *Paramecium* (Cronkite, 1979; Hiwatashi, 1981) such as that of fertilization in metazoan eggs (Mazia, 1937; Monroy, 1947; Epel, 1980). If no change in membrane potential was observed after application of the LIS-MV as mentioned before, rapid influx of Ca^{2+} into the cell might not occur during the mating reaction. The above conclusion agrees well with the facts that formation of conjugating pair by the mating reaction is not inhibited by the Ca-channel blocker $LaCl_3$ (Cronkite, 1977) and natural conjugation occurs in all known mutants with defective Ca-channels (Cronkite, 1974; Takahashi, 1979). Therefore, a membrane potential change such as the fertilization potential which functions as a fast block to polyspermy in echinoderms (Jaffe, 1976; Miyazaki and Hirai, 1979), echiuriens (Gould-Somero *et al*, 1979), and amphibians (Cross and Elinson, 1980; Grey and Schertel, 1978) appears unnecessary for the activation of conjugation in *Paramecium*.

The mating-type-specific ciliary inactivation or decrease in swimming velocity reported here can be used as a good method for assaying the mating substances since it is a very simple and clear indicator of activation of conjugation in *Paramecium*.

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PHYSIOLOGY OF THE WOOD BORING MOLLUSC *MARTESIA CUNEIFORMIS* SAY

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ABSTRACT

Larvae of *Martesia cuneiformis* Say settled in greatest abundance (151.5 individuals/square meter surface area of wood substrate) during the months of August, September, and October in a North Carolina estuary. Minimum estimates of post settlement shell growth rates are 0.19 mm/day during the summer months and 0.03 mm/day during the winter months. Gravid adults maintained in the laboratory spawn during physical disturbance of dissection from wood and, in isolated individuals, after thermal stimulation. The first shelled veliger larva has a mean shell size of 69.0 μm long and 60.0 μm high. At 22°C and 30–32‰ salinity the pediveliger stage, with a mean shell size of 260 μm long and 269 μm high, is reached in 35 days. Larval respiration rate is described by the relationship $R = 2.22W^{2.42}$ where R is oxygen consumption (nl O_2/larva/h) and W is dry weight (μg). On starvation larvae predominantly utilize protein and lipid as respiratory substrates. Adult respiration and ammonia excretion rates are described by the relationships $R = 17.95W^{0.703}$ and $E = 0.133W^{0.492}$ respectively with the units R (μl O_2/individual/h), E (μg at NH_3-N/individual/h), and W (g live weight).

INTRODUCTION

Bivalve molluscs of the family Pholadidae are unusual in that, in the adult form, they bore into a variety of substrates including sand, peat, clay, wood, calcareous shells of other molluscs, and rocks varying in hardness from sandstone and limestone to chert and shale. Certain aspects of the biology of the Pholadidae are reasonably well documented. They have a bathymetric range varying from the intertidal and shallow subtidal (Turner, 1954) to opportunistic occurrence in woody substrates in the deep sea (subfamily Xylophagainae, see Turner, 1973). The taxonomy of the members of the family has received considerable attention (Turner 1954, 1955) and remains a subject of active research. The larvae of a number of species have also been cultured and described (Lebour, 1938; Werner, 1939; Jorgensen, 1946; Rees, 1950; Chanley, 1965; Chanley and Andrews, 1971; Boyle and Turner, 1976). By contrast data on the physiology of both the larval and adult forms, with exception of work on adult *Martesia fragilis* (Verrill and Bush) and in one instance *Martesia striata* Linné (Nagabushnam, 1961; Srinivasan, 1961, 1962, 1963a, b, c; Srinivasan and Krishnaswamy, 1964) are rare.

As part of a larger study of bioenergetics of wood boring molluscs of the family Teredinidae the opportunity arose to collect adult pholads, *Martesia cuneiformis* Say. This report describes subsequent studies in which these adults were maintained and induced to spawn in the laboratory. The larvae were cultured to metamorphosis and used to examine aspects of respiration and change in biochemical composition during

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growth and starvation. The relationship of oxygen consumption to nitrogenous excretion of adult animals was also examined.

MATERIALS AND METHODS

Seasonality of settlement for *Martesia cuneiformis* larvae was determined for the Beaufort, North Carolina region by suspending fir panels (15 × 9 × 4 cm = 0.462 m^2) below mean low water off the dock of the Duke University Marine Laboratory. Ten groups of 10–12 panels each were immersed for varying lengths of time (2–6 months) over a three year period. On retrieval panels were cleaned of attached macrofauna, wrapped in wet paper, packed in an insulated container, and shipped to Woods Hole, Massachusetts. Panels were subsequently maintained in flowing, coarsely filtered (100 μm) sea water between 10–16°C and at ambient salinity of 30–32‰. Number and location of *M. cuneiformis* within each panel was documented by x-radiography. Individual *M. cuneiformis* were dissected from panels and maintained as above. Spontaneous spawning of gravid adults commenced upon dissection from the wood or was initiated in isolated individuals by thermal stimulation at 30°C for one hour in static, 1 μm filtered sea water at 30–32‰ salinity.

Eggs were collected and rinsed on a 20 μm "Nitex" nylon mesh, and fertilization subsequently effected in 3 l glass jars at a density of 50 eggs/ml of 0.45 μm filtered sea water equilibrated to 22 ± 2°C. Trochophore and subsequent shelled larvae were cultured in a 50 l cylindrical polyethylene (Nalgene) container at a density of 1–10/ml (decreasing from the highest density at early stages to lowest density prior to metamorphosis). Water was changed every two days at which time the larvae were siphoned on to a suitable size "Nitex" mesh and rinsed with filtered sea water. The culture container was thoroughly washed, refilled with filtered sea water, supplied with the flagellate *Isochrysis* aff. *galbana* (clone T-ISO) to a concentration of 5 × 10^4 cells of food per ml of final culture volume, and larvae returned to the container.

The culture was provided with continuous gentle aeration. At each water change a subsample of larvae was removed, fixed in 10% buffered formalin, and subsequently used to assay growth of the cultured larvae. This was recorded as length (anterior-posterior axis) and height (dorsal-ventral axis) on a minimum of 20 individuals from each sequential sampling. All measurements were made with a Leitz compound microscope fitted with an ocular micrometer.

All measurements of larval respiration and biochemical composition were made on larvae from one culture. Larval respiration was assayed using the differential respirometer of Grunbaum *et al.* (1955). Respirometer flasks had a volume of about 5 ml. Assays used between 100 and 1000 larva, decreasing in number with increasing size of the larvae, held in 2 ml of 0.45 μm filtered sea water in the respirometer flask. All assays were made in the absence of food organisms. Manometric readings were made at 30 minute intervals for periods of up to four hours on any one group of larvae. Computations of respiration rate used only initial and final readings. On completion of an assay larvae were fixed in 10% buffered formalin to await length and weight measurement as described earlier. Blank assays without larvae provided a control for bacterial respiration.

On each of days 15 and 35 after fertilization two extra subsamples of larvae were taken. One subsample was quickly rinsed in 3% w/v ammonium formate, washed into a clean glass vial, deep frozen, freeze dried, and subsequently stored in a sealed vial in a dessicator to await analysis. The second subsample of larvae was starved in 0.22 μm filtered sea water, under otherwise identical conditions to the actively growing larvae, for a period of three days, and then prepared for biochemical analysis as described earlier. The analytical procedures used were as follows:

(i) Individual mean dry weights were determined from weights of a known number of freeze dried larvae using a Perkin Elmer microgram balance.

(ii) Ash weight was determined from weight of the above after ignition to constant weight at 450°C. Ash-free dry weight is the difference between dry weight and ash weight.

(iii) Carbon and nitrogen contents were determined using a Perkin Elmer Carbon-Hydrogen-Nitrogen (CHN) elemental analyzer. Protein is expressed as nitrogen × 6.25.

(iv) Carbohydrate and lipid contents were determined on homogenates of known weights of freeze dried larvae in distilled water. Homogenates were prepared using a Branson sonifier. Lipid assay used a first extraction in 1:2 v/v chloroform: methanol (Bligh and Dyer, 1959), a second extraction in 2:1 v/v chloroform: methanol (Folch *et al.,* 1957), purification with 0.7% w/v sodium chloride (Marsh and Weinstein, 1966), drying of the chloroform layer, and gravimetric analysis. Carbohydrate assay used 5% cold trichloroacetic acid to precipitate protein and nucleic acids from the initial water homogenate (Holland and Gabbott, 1971). Carbohydrate content of supernatant was assayed by the phenol-sulphuric acid method of Raymont *et al.* (1963) using glucose as a standard. Reported values are mean values of triplicate analyses.

Estimates of caloric content were made from biochemical composition using the following conversion factors: protein (nitrogen × 6.25) 5.7 cal/mg, carbohydrate 4.2 cal/mg, and lipid 9.5 cal/mg (Ansell, 1972).

All measurements of respiration and excretion by adult *Martesia cuneiformis* were made on individuals dissected from the wood. Oxygen consumption was measured using a Gilson differential respirometer. During assay *M. cuneiformis* were held in filtered sea water. Pumping of water by the animals made agitation of the respirometer flasks unnecessary. Concurrent measurements were made, at the beginning and end of the assay period, of ammonia and dissolved primary amine concentration in the water containing the individual *M. cuneiformis* using the phenol hypochlorite method of Solorzano (1969) and the fluorescamine method of Undenfriend *et al.* (1972) as modified by North (1975) respectively. A minimum of two respirometer flasks containing filtered water only were included in each experimental assay to provide blank measurements of ammonia and primary amine concentrations. The fluorescamine method was calibrated against glycine. All measurements on adult animals were made within seven days of their being dissected from the collecting panel.

RESULTS

Larval settlement is expressed as individual larvae per square meter of surface area of the wooden substrates. No settlement was recorded during January, February, and March. Settlement occurred at densities of 3.6 ind./sq. m during June and July, 151.5 ind./sq. m during August, September, and October, and 45.1 ind./sq. m during November and December.

From a total of 120 panels examined 73% of the larvae metamorphosed on the cut end grain of the wood panels, 18% on the edges and 9% on the relatively smooth, flat sides of the panels.

Upon removal from the wood, shell length, defined as the maximum anterior-posterior dimension of the valves, was recorded. Estimates of daily increment of shell length were made. These varied from 0.04–0.19 mm/day during the months of August, September, and October to 0.01–0.03 mm/day during December, January, and February respectively. These are probably underestimates of the actual growth rate since they are based on the assumption that larval settlement occurred on day one of substrate immersion.

Fifty individuals were dissected from fir panels that had been immersed in sea water from 30/6/80 to 24/10/80 (116 days). These varied in length from 5 to 22 mm with a mean value of 11.7 mm. Twenty four of these individuals spawned, fourteen as males (length range 5–19 mm, mean 11.9 mm), nine as females (length range 10–16 mm, mean 11.7 mm), and one individual, of length 16 mm, as a male on one occasion and twice within the following four days as a female. Spawning activity was not related to development of the callum in that one individual with no callum spawned as a female upon removal from the wood, and a further seven individuals with no or only a partly developed callum simultaneously spawned as males. Neither was there a strong relationship between shell length and the state of development of the callum in collected specimens. Individuals with no or only a partly developed callum varied in length from 5 to 19 mm (mean = 10.3, n = 25) whereas those with a full callum varied in length from 7 to 22 mm (mean = 13.1, n = 25). No mortalities were observed as a direct consequence of dissection from the wood. Isolated pholads were maintained in flowing sea water (12–18°C) for over one year and retained the ability to spawn upon stimulation.

The first shelled veliger larva has dimensions of 69.0 ± 2.8 μm S.D. length and 60.0 ± 3.0 μm S.D. height (n = 20). From 12 to 25 days post fertilization the larva

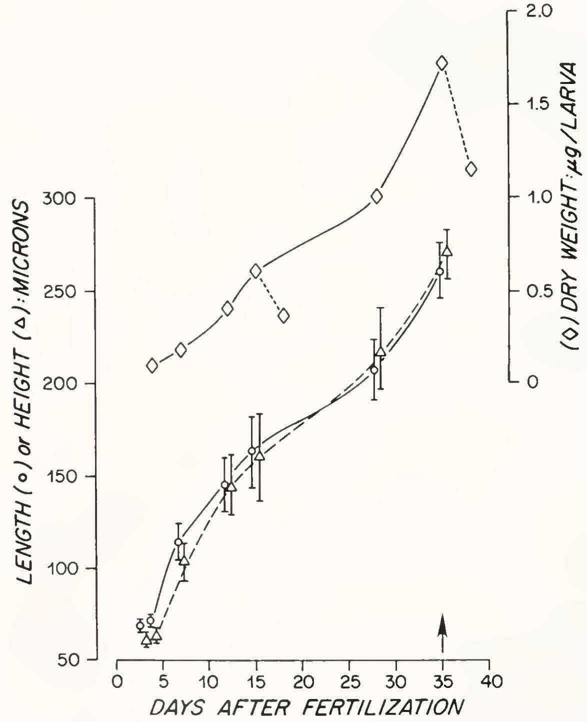

FIGURE 1. Change in length (○ —— ○), height (△ —— △) and dry weight inclusive of shell (◇ —— ◇) of *Martesia cuneiformis* throughout larval development at 22°C on a diet of *Isochrysis* aff. *galbana* (clone T-ISO). Weight loss during starvation indicated thus (◇ - - - ◇). Error bars, where given, are ±1 S.D., n = 20. Arrow on x axis indicates 50% of population are pediveliger larvae.

is approximately isodiametric (Fig. 1). The pediveliger larval foot appears at a mean shell length of 260 μm. Approximately 50% of the population reach pediveliger stage 35 days after fertilization. The dry weight (including shell) of the larva increases steadily from a mean value of 0.1 μg at 4 days post fertilization to 1.72 μg at 35 days post fertilization. Marked decreases in dry weight resulted from three day starvation periods that began on days 15 and 35 respectively (Fig. 1).

Oxygen consumption rate per larva increased allometrically with dry weight of the larva including shell (Fig. 2). The relationship is described by the equation:

$$R = 2.22W^{2.42}$$

where R is the respiration rate (nl O_2/larva/h) and W is the dry weight including shell (μg).

Marked decreases in ash free dry weight were observed during starvation (Table I). Starvation losses in a three day period amounted to approximately two thirds of the prestarvation protein and lipid contents by weight, and over three quarters of the prestarvation carbohydrate content by weight. Although absolute losses by weight were consistently highest in the protein fraction and lowest in the carbohydrate fraction the relative contributions of these fractions to energy expenditure are best examined in terms of caloric equivalents (Table I). Protein was the major contributor to energy expenditure during starvation accounting for 56 and 54% of total caloric losses during

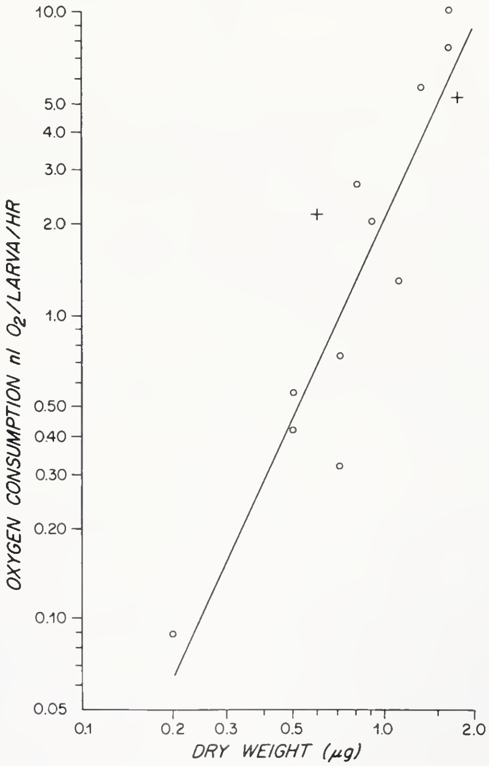

FIGURE 2. Double logarithmic plots of respiration rate and dry weight inclusive of shell for larval *Martesia cuneiformis*. Line fitted to recorded values (O): $R = 2.22W^{2.42}$, $r = 0.928$, $n = 11$, $P < 0.01$. Values recorded (+) are calculated from caloric loss during starvation (see Table I).

TABLE I

Biochemical composition of Martesia cuneiformis *larvae grown at 22 ± 2°C and 30–32‰ salinity on a diet of* Isochrysis *aff.* galbana *(clone T-ISO)*

Days after fertilization	15	18	35	38
Days fed	15	15	35	35
Days starved	0	3	0	3
Length μm	162.6		260.4	
Height μm	160.8		269.4	
Dry wt: μg/larva	0.60	0.36	1.72	1.14
Ash free dry weight: μg/larva	0.308	0.087	0.946	0.463
Biochemical content: μg/mg dry wt				
carbohydrate	10.71	3.53	25.5	8.00
lipid	80.0	47.0	59.0	30.1
nitrogen × 6.25	172.5	94.3	293.7	162.5
carbon	205.0	174.0	260.0	188.0
Biochemical content: ng/larva				
carbohydrate	6.43	1.27	43.86	9.12
lipid	48.00	16.92	101.48	34.20
nitrogen × 6.25	103.5	33.9	505.2	185.2
carbon	16.6	62.6	214.3	80.8
Biochemical content: relative contribution as calories × 10^{-6}/larva				
carbohydrate	27.0	5.3	184.2	38.3
lipid	456.0	167.7	964.0	324.9
nitrogen × 6.25	589.9	193.2	1375.4	460.6
total	1072.9	359.2	2523.6	823.8

starvation regimes initiated on days 15 and 35 respectively. By comparison lipid and carbohydrate contributed 40 and 3% of total energy expenditure respectively during the starvation regime initiated on day 15, and 38 and 9% of total energy expenditure respectively for the regime initiated on day 35.

Oxygen consumption rate increased allometrically with increasing weight in adult *Martesia cuneiformis* (Fig. 3) and is described by the relationship:

$$R = 17.95W^{0.703}$$

where R is the respiration rate (μl O_2/individual/h) and W is the live weight (grams). Ammonia excretion rate also increased allometrically with increasing weight (Fig. 3) according to the relationship.

$$E = 0.133W^{0.492}$$

where E is the excretion rate (μg at NH_3-N/individual/h) and W is the live weight (grams). Fifteen measurements of excretion of amine nitrogen were made. Results indicated a consistently low excretion rate with amine nitrogen contributing a mean of 2.8% of the total nitrogen excreted.

The ratio of oxygen consumption to total nitrogen excretion on individual assays varied between 6.8 and 24.4 with a mean value of 12.1. Despite the differences in the exponent values of the allometric relationships for excretion and respiration rates *versus* weight the O:N ratio did not appear to be closely related to the weight of the animal.

DISCUSSION

Martesia cuneiformis is a hermaphrodite possibly maturing protandrically initially as a male and subsequently as a female. A similar observation has been recorded by

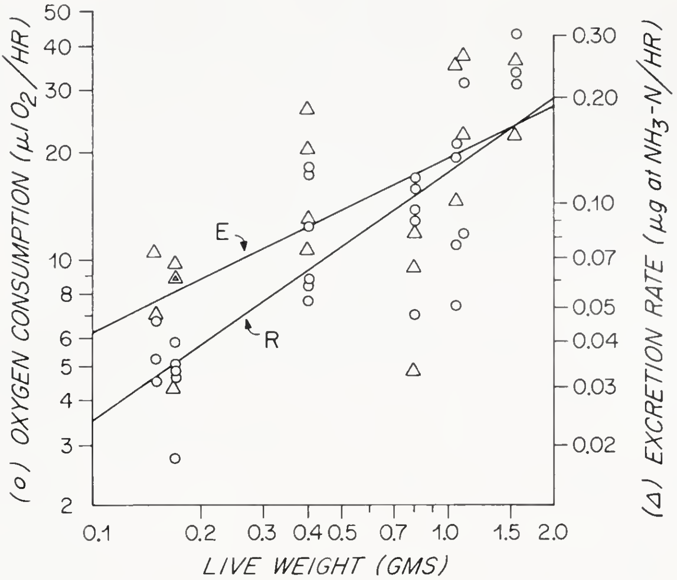

FIGURE 3. Double logarithmic plots of respiration rate (O) and ammonia excretion rate (△) *versus* live weight for individual adult *Martesia cuneiformis*. $R = 17.95W^{0.703}$ ($r = 0.827$, $n = 29$, $P < 0.01$); $E = 0.133W^{0.492}$ ($r = 0.645$, $n = 19$, $P < 0.01$).

Srinivasan and Krishnaswamy (1964) for *Martesia fragilis*. The gonad of *M. cuneiformis* can develop rapidly as the functional foot degenerates and spawning can occur prior to the development of a complete callum.

The major peak of larval settlement in the Beaufort, North Carolina area apparently occurs during late summer and early fall. No comparable data could be found on this or similar pholad species; however, this data is in contrast to that for larval settlement of the Teredinid *Bankia gouldi* Bartsch which peaks in late spring and early summer at the same location (Mann and Gallager, unpubl.). The greatest larval settlement density of 151.5 individuals/sq. m for *Martesia cuneiformis*, is also substantially lower than that of *B. gouldi* (*i.e.*, 8500 ind./sq. m in June; Mann and Gallager, unpubl.). A lower fecundity in *M. cuneiformis* and crowding of the substrates by late summer may be controlling factors of competition for space.

The maximum shell growth of 5.7 mm/month for *Martesia cuneiformis* is typical for this species with *Martesia striata* growing slightly faster at similar temperatures (Turner, per. comm.).

Spawning of gravid *Martesia cuneiformis* in the laboratory was observed only in response to physical disturbance during dissection from the wood or after thermal stimulation. This is unlike *Martesia striata* which spawns spontaneously at 21°C (Boyle and Turner, 1976). The first shelled veliger of *M. striata* is, at 68.0 µm long and 59.1 µm in height, of similar size to the first shelled veliger of *M. cuneiformis*. The pediveliger of *M. striata* at 235.7 µm long and 236.2 µm in height, is, however, smaller than that of *M. cuneiformis*. Boyle and Turner (1976) describe a tooth on one valve and a corresponding notch on the other along the ventral margin of *M. striata* pediveligers. This morphological feature does not exist in *M. cuneiformis*. Growth rate of *M. cuneiformis* larvae at 22°C is comparable to that of *M. striata* at 26°C (Fig. 1, and Boyle and Turner, 1976) but slower than reported for *Teredo navalis* Linné (Gallager and Mann, 1981a), *Crassostrea virginica* Gmelin (Dupuy *et* *al.*, 1978), *Ostrea edulis* Linné (Walne, 1965), or *Mytilus edulis* Linné (Bayne, 1976) under comparable conditions of temperature, salinity, and food concentration.

The respiration rate of *Martesia cuneiformis* larvae is notably lower than those reported previously for *Crassostrea virginica* by MacInnes and Thurberg (1973). The exponent value 2.42 relating weight to oxygen consumption is considerably higher than the value of 0.902 recorded for *Mytilus edulis* veligers by Riisgard *et al.* (1981), and higher than values suggested by Zeuthen (1953) for small marine organisms.

During starvation of *Martesia cuneiformis* larvae energetic losses are greatest in the protein component with a significant loss also being recorded in the lipid component (Table I). In contrast Holland and Spencer (1973) working with *Ostrea edulis*, and Gallager and Mann (1981a) working with *Teredo navalis*, both emphasize the importance of neutral lipid as the predominant energy reserve during enforced starvation of the larval stages. In general, close agreement exists between the composition data on a μg/mg dry weight basis for the larvae of *M. cuneiformis* (Table I) with those of *Ostrea edulis* of comparable size (Holland and Spencer, 1973).

The measurement of both respiration rate and change in biochemical content during starvation of *Martesia cuneiformis* larvae allows comparison of observed oxygen consumption with that calculated from oxycaloric equivalents. Calculated caloric losses during three day starvation periods initiated on days 15 and 35 respectively are 713.7×10^{-6} and 1699.8×10^{-6} cal/larva respectively (Table I), or 9.91×10^{-6} and 23.61×10^{-6} cal/larva/h respectively assuming a constant rate of depletion of reserves over the three day period. Oxygen consumption rates of 15 and 35 day old larvae are 0.64 nl/larva/h and 8.25 nl/larva/h respectively. These values give calculated oxycaloric equivalents of 15.48 cal/ml O_2 and 2.86 cal/ml O_2 respectively. When compared with respiration values calculated from biochemical component loss (Table I) and oxycaloric equivalents for individual components of 4.94 cal/ml O_2 for carbohydrate, 4.59 cal/ml O_2 for lipid, and 4.48 cal/ml O_2 for protein degraded to ammonia (values recalculated from Elliot and Davison, 1975) the recorded values suggest under and over estimation of oxygen consumption for *M. cuneiformis* larvae of 162.6 and 260.4 μm length respectively from the fitted line in Figure 2. Such an error in estimation could result from a combination of high exponent value and a low intercept value. For comparison purposes respiration rate measurements estimated from caloric losses, that is 2.16 nl/larva/h and 5.18 nl/larva/h respectively, are plotted on Figure 2.

A good agreement is noted in the exponent value of 0.703 relating wet weight to respiration rate for adult *Martesia cuneiformis* with exponent values of 0.68 for *Teredo navalis* at 21°C (Soldatova, 1961a, b), 0.76 for *Lyrodus pedicellatus* Quatrefages at 20°C (Gallager *et al.*, 1981b), 0.78 for *Mytilus edulis* at 20°C (Gallager *et al.*, 1981b), and 0.611 for *M. edulis* at 15°C (Bayne and Scullard, 1977). Definitive comparisons of absolute respiration rate are not possible because no measurements were made of dry tissue weight of the individual *M. cuneiformis*. Previously Gallager and Mann (1981b, Table III) have shown that dry tissue weight varies between 7.48 and 8.67% of the live weight of fed *Tapes japonica* (Deshayes) in the size range 1.25–1.73 g live weight. If a mean of the values calculated from Gallager and Mann (1981b) is applied to *M. cuneiformis* the relationship plotted in Figure 3 becomes:

$$R \text{ (ml } O_2\text{/individual/h)} = 0.264W^{0.703}$$

where W is the dry tissue weight in grams. While the validity of this estimate is limited by the application of data from *T. japonica* to *M. cuneiformis* it is notable that the value of the constant obtained, that is 0.264, is comparable to those recorded for the byssaly attached *Mytilus edulis* (0.302 and 0.398 by Bayne and Scullard, 1977; and

Gallager *et al.*, 1981b respectively) but considerably lower than those recorded for actively boring teredinids (1.20 by Soldatova, 1961a, b; and 1.313 by Gallager *et al.*, 1981b). Gallager *et al.* (1981a) previously showed that respiration rate increases approximately four fold on transition from quiescence to active boring in the teredinid *Bankia gouldi*. It is probable that similar increases occur in adult *M. cuneiformis* during active boring in a wood substrate.

The exponent value of 0.492 which relates ammonia excretion rate to wet weight of individual adult *Martesia cuneiformis* is lower than the corresponding exponent for respiration. Bayne (1976, Table 6.5) summarizes data on seasonal changes in the exponent for excretion rate in *Mytilus edulis* and shows it to vary between 0.35 (starved animals in winter) and 1.20 (fed animals in winter). The value recorded in the present study is comparable to those recorded by Mann (1979a) for *Crassostrea gigas* at 12–18°C and *Ostrea edulis* at 12–15°C. The low contribution of amine nitrogen to the total excreted nitrogen is not typical of other reports in the literature. Hammen (1968) reported contributions of amine nitrogen to the total excreted by *Tagelus plebius* that varied from 31% (normal) to 67% (stressed animals). Similarly Bayne (1973) found that the proportion of amino nitrogen excreted increased from 55 to >75% during temperature and starvation stress in *M. edulis.* Recent criticism of the fluorescamine technique suggests that comparison of the present data with literature values obtained by other methods is difficult. Wright (1982) states that fluorescamine may react with primary amines other than amino acids; *e.g.*, ammonia, thus introducing an overestimation of the contribution of amines to total nitrogenous excretion; however, even if this overestimation was made in the present study the observation concerning proportionately low amino acid excretion in *M. cuneiformis* still remains valid.

The significance of the ratio of oxygen consumption to ammonia excretion as an indicator of predominant respiratory substrate in marine bivalves has been discussed at length in Bayne (1976, pp. 268–270). The values recorded for *M. cuneiformis* are low and indicate a strong dependence on protein. Biochemical component assays of adult animals were not made to substantiate this conclusion; however, the individual animals on which O:N ratios were recorded had well developed callums, were reproductively active or both. Gametogenesis often proceeds at the expense of stored lipid and carbohydrate reserves in marine molluscs (see Gabbott 1975, Mann 1979a, b) and the post-spawning period is often marked by a notable increase in ammonia excretion rate as protein reserves are utilized more intensively (Mann, 1979a, b). It is not, therefore, unreasonable to suggest that the low O:N ratio recorded here is a result of prolonged spawning activity and depletion of reserves.

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D-GALACTOSIDE SPECIFIC LECTINS FROM COELOMOCYTES OF THE ECHIURAN, *URECHIS UNICINCTUS*

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ABSTRACT

Two lectins specific for D-galactosides with molecular weights of 31,000 and 34,000 (estimated by SDS-PAGE, under reduced conditions) were purified from coelomocytes of the echiuran, *Urechis unicinctus.* They were eluted together from a Bio-Gel P-100 column as a single peak with an apparent molecular weight of 35,000. They closely resembled each other in saccharide specificity, isoelectric point, and electrophoretic mobility under unreduced conditions. The hemagglutinating activity of both lectins was inhibited by a glycoconjugate fraction obtained from the Pronase-digest of coelomocytes.

INTRODUCTION

Many kinds of lectins have been identified and purified from a variety of animals including sponges (Bretting and Kabat, 1976; Müller *et al.,* 1979), snails (Hammarström and Kabat, 1969; Van der Knaap *et al.,* 1982), horseshoe crabs (Marchalonis and Edelman, 1968), insects (Komano *et al.,* 1980; Suzuki and Natori, 1983), sea urchins (Ryoyama, 1974; Sasaki and Aketa, 1981; Yamada and Aketa, 1982), fish (Teichberg *et al.,* 1975), frogs (Roberson and Barondes, 1982), chicks (Nowak *et al.,* 1977), and mammals (Hudgin *et al.,* 1974). Animal lectins are thought to participate in various physiological events such as clearance of blood components by the liver (Ashwell and Morell, 1974), adhesion of sponge cells (Müller *et al.,* 1979), differentiation of limb bud cells (Matsutani and Yamagata, 1982), infection of bacteria (Eshdat *et al.,* 1978), fertilization of *Xenopus* eggs (Wyrick *et al.,* 1974), transport and storage of sugar (Sharon and Lis, 1972), and self-defense in insects (Komano *et al.,* 1980).

The self-defense mechanisms or "immune systems" in invertebrates have been attributed mostly to coelomic fluid (hemolymph). Coelomocytes agglutinate or clump each other in response to wounding (Boolootian and Giese, 1959). The coelomic plasma contains hemagglutinins (Cushing *et al.,* 1963; Brown *et al.,* 1968; Acton and Weinheimer, 1975) or antibacterial activities (Bang and Krassner, 1958; Johnson and Chapman, 1970). However, there is little biochemical information on the echiuran coelomic fluid, although it is easily collected in large quantities (Dybas, 1981). Echiuran coelomocytes also clump if they are taken out of the body. There is no definitive evidence indicating any bactericidal activities in the coelomic fluid of echiurans. We have searched for echiuran lectins which possibly may be involved in the self-defense mechanism. This paper describes the purification and characterization of two lectins specific for D-galactoside in the coelomocytes of *Urechis unicinctus.*

MATERIALS AND METHODS

Preparation of crude lectin

Echiuroid worms, *Urechis unicinctus,* were collected at Choshi, Chiba Prefecture, and at Matsuyama, Ehime Prefecture, and kept in an aquarium at 6–10°C until used.

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Purification of lectin was carried out at 4°C unless otherwise indicated. Animals were bled by cutting the body wall and the combined coelomic fluid was centrifuged at 1000 × *g* for 10 min. Coelomocytes were washed with Ca^{2+}-Mg^{2+} free sea water (500 m*M* NaCl, 9 m*M* KCl, 29 m*M* Na_2SO_4, 2 m*M* $NaHCO_3$) to eliminate germ cells, and were suspended in 10 volumes of the extraction medium; 200 m*M* lactose in medium A (0.05% Triton X-100, 10 m*M* 2-mercaptoethanol, 150 m*M* NaCl, 10 m*M* EDTA, 5 m*M* benzamidine, 10 m*M* Tris-HCl, pH 7.4). The cells were sonicated six times for 20 s at 0°C (Tomy Seiko model UR-200P, 110W) and centrifuged at 27,000 × *g* for 30 min. The supernatant was passed through a sheet of filter paper to remove floating lipids. Ammonium sulfate was gradually added to the extract to 50% saturation and the mixture was gently stirred for 4 h. The precipitate was removed by centrifugation at 27,000 × *g* for 1 h, and the concentration of ammonium sulfate was increased up to 90% saturation. The precipitate was collected by centrifugation at 27,000 × *g* for 30 min, and dissolved in 10 ml of medium A. The solution was extensively dialyzed against medium A for 48 h to eliminate lactose. The crude extract thus obtained was stored at −20°C until used.

Hemagglutination assay

Trypsinized and glutaraldehyde-fixed rabbit erythrocytes were used for standard assays. Human, swine, and bovine erythrocytes, either fresh or fixed, were also used for the test of erythrocyte specificity. Fresh erythrocytes were washed several times with medium B (10 m*M* EDTA, 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4). Trypsinized and glutaraldehyde-fixed cells were prepared according to the method of Sasaki and Aketa (1981). Since the preliminary experiment showed that Triton X-100 prevented non-specific adhesion of erythrocytes to titer plates, the hemagglutinating activity was assayed in 0.025% Triton X-100. Test solutions (25 μl) of serial 2-fold dilutions and 2% suspension of erythrocytes (25 μl) were mixed in each well of a microtiter V-plate (Cooke Engineering). The hemagglutination was scored after the plates were kept in a moisture chamber for 1 h at 20°C. The activity was expressed by titer defined as the reciprocal of the highest dilution giving positive hemagglutination. The effect of various saccharides on hemagglutination by the lectin was studied by the addition of serially diluted saccharide solutions to the hemagglutinating assay mixture containing lectin of titer 8. Inhibitory activities were expressed as minimal concentrations of saccharides to inhibit the activity of lectin. D-Thiodigalactoside (D-galactopyranosil-β-D-thiogalactopyranoside), methyl-β-D-thiogalactopyranoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside, D-galactose, N-acetyl-D-galactosamine, methyl-β-D-xyloside, α-D-fucose, melibiose (D-glucopyranosil-β-1,6-D-galactopyranoside), fetuin, and heparin were purchased from Sigma Chemical Company. Lactose (D-glucopyranosil-β-1,4-D-galactopyranoside) was purchased from DIFCO Laboratories. L-Arabinose and D-glucose were purchased from Wako Chemicals. Glycogen and L-fucose were purchased from Katayama Chemicals.

Affinity chromatography

Lactose was conjugated to the epichlorohydrin-activated Sepharose 6B as follows. Sepharose 6B (120 ml) (Pharmacia Fine Chemicals) was washed well with distilled water (DW) and stirred in the mixture of 80 ml of 1 *M* sodium hydroxide and 10 ml of epichlorohydrin (Katayama Chemicals) for 24 h at 20°C. Activated Sepharose 6B was washed several times with DW, and was resuspended in 200 ml of 2 *M* sodium carbonate buffer (pH 10) containing 8 g of lactose. The suspension was incubated for 3 days at 20°C with shaking. The lactose-conjugated Sepharose 6B was extensively washed with medium A and stored at 4°C until used.

About 10 ml of crude lectin extract was slowly applied to a lactose-Sepharose 6B column (2.8 × 7.5 cm). Unadsorbed materials were washed out with 150 ml of medium A. The material adsorbed to the column was eluted with medium A containing 100 m*M* lactose. Effluents were monitored at 280 nm. Each fraction of 2.5 ml was extensively dialyzed against medium A. The fractions which had hemagglutinating activity were combined and used for general lectin characterization.

Gel filtration

A column of Sephadex G-100 (1.3 × 70 cm, Pharmacia Fine Chemicals) and a column of Bio-Gel P-100 (1.3 × 52 cm, Bio Rad Laboratories) equilibrated with medium A containing 100 m*M* lactose, were used for the molecular weight estimation of native lectins. Each fraction of 0.8 ml was dialyzed against medium A to eliminate lactose and assayed for the hemagglutinating activity. In some experiments, medium A, 200 m*M* lactose in medium A, and 50 m*M* lactose-200 m*M* glucose in medium A were also used as the elution media. Bovine serum albumin (68,000), ovalbumin (43,000), deoxyribonuclease I (31,000), α-chymotrypsinogen A (25,000), and myoglobin (17,000) (Sigma Chemical Company) were used as molecular weight markers.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) using a mini-slab gel apparatus (8.4 × 9.2 cm) was performed according to Laemmli (1970) but with slight modifications. After electrophoresis, protein bands were revealed by silver staining (Wray *et al.*, 1981).

Preparative SDS-PAGE. The active fraction from the lactose-sepharose 6B column was diluted with an equal volume of sample buffer (150 m*M* Tris-HCl, pH 6.8, 0.2% SDS, 20% glycerol, 2% 2-mercaptoethanol). Aliquots of 10–20 μl were applied to the wells without heating. Electrophoresis was performed using a buffer system of 0.38 *M* glycine, 25 m*M* Tris, 0.1% SDS, and 1% 2-mercaptoethanol (pH 8.3) at 10 mA per plate. Both stacking gel (5% acrylamide) and separating gel (10% acrylamide) contained 0.1% SDS. After electrophoresis, the gel was cut into three pieces; the side pieces were used for silver staining, and the central one for purification. The central piece of the gel was sliced 1 or 2 mm thick, and each slice was extracted with 300 μl of medium A overnight at 0°C. An aliquot of each extract was reexamined for the purity by the analytical SDS-PAGE, and another aliquot was assayed for the hemagglutinating activity after extensive dialysis against medium A.

Analytical SDS-PAGE. Sample solutions were mixed with an equal volume of sample buffer (150 m*M* Tris-HCl buffer, pH 6.8, 2% SDS, 20% glycerol, 5% 2-mercaptoethanol) and the mixtures were heated at 90°C for 3 min. The samples were subjected to electrophoresis as described above.

Isoelectric focusing (IEF)

Polyacrylamide gel (5%) containing 2% Ampholine (LKB; pH 4–6) and 0.05% Triton X-100 was prepared in tubes (0.5 × 8 cm). Sodium hydroxide (20 m*M*) and phosphoric acid (10 m*M*) were used as the cathode and the anode solution, respectively. Focusing was performed at a fixed current (1 mA/tube) for 1 h, and then at a fixed voltage (400 V) for 5 h. After focusing, the gel was sliced and extracted as above.

Enzyme treatments

Lectin preparations after affinity chromatography were incubated with one of the following enzymes at its optimal pH at 25°C for various periods; trypsin (0.1 mg/ml, Sigma Chemical Company), Pronase P (0.1 mg/ml, Kaken Chemicals), protease from *Streptomyces griseus* (0.1 mg/ml, Sigma Chemical Company), α-chymotrypsin (0.1 mg/ml, Sigma Chemical Company), alkaline phosphatase (50 units/ml, Boehringer Mannheim), and glycosidase mixture from *Turbo cornutus* (0.1 mg/ml, Seikagaku Kogyo). After enzyme treatment, remaining activities were assayed at 4°C. For control experiments, enzyme solutions were replaced with the same volume of medium A.

Pronase digestion of coelomocytes

The residues after lectin extraction of coelomocytes were resuspended in 100 ml of DW and dialyzed against 2 liters of DW with 3 changes for 3 days. The dialysate was mixed with 100 ml of 0.1 *M* borate buffer (pH 8.0) containing 80 mg of Pronase P, 10 m*M* $CaCl_2$, and 0.02% sodium azide. After addition of small volume of toluene, the mixture was incubated at 37°C for 48 h with gentle shaking. The digestion by Pronase was stopped by adding trichloroacetic acid to a final concentration of 10% (w/v). The supernatant obtained after centrifugation was neutralized with 10 *N* sodium hydroxide, and was concentrated to 25 ml with a rotary evaporater at 30°C. After centrifugation of the concentrates at 27,000 × *g* for 30 min, 5 ml of the supernatant was fractionated with a Sephadex G-25 column (medium, 2.7 × 87 cm), equilibrated and eluted with 50 m*M* pyridine acetate buffer, pH 5.2. The break through fractions were concentrated with a rotary evaporater. The concentrate was dialyzed against medium A and assayed for the inhibition of the hemagglutinating activity.

Heat stability test and chemical analysis

The lectins in medium A (20 μg protein/ml) were incubated at 20, 40, and 60°C. At various time intervals, aliquots were assayed for the hemagglutinating activity at 4°C.

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. Saccharide concentration was assayed by the phenol-sulfuric acid method (Dubois *et al.,* 1956), using glucose as a standard.

RESULTS

Extraction and purification of the lectins

A high hemagglutinating activity was detected only when coelomocytes were disrupted, while it was not detected in the coelomic plasma or in the suspension of intact coelomocytes. The activity in the cells was effectively solubilized by 100 m*M* lactose in the medium. After extraction with this medium, the activity was not detectable in the residue, indicating the apparent solubilization of 100% activity. In the absence of lactose, however, only 3% of the activity was solubilized.

The activities solubilized in the absence and in the presence of lactose differed from each other in some properties. The activity solubilized by the lactose-free medium was precipitated by ammonium sulfate at 50% saturation, and was not adsorbed to the lactose-Sepharose 6B column. On the contrary, most of the activity solubilized by the medium containing lactose precipitated between 50–90% saturation of ammonium sulfate and was readily adsorbed to the lactose-Sepharose 6B column. The activity adsorbed to the column was quantitatively eluted by 100 m*M* lactose as

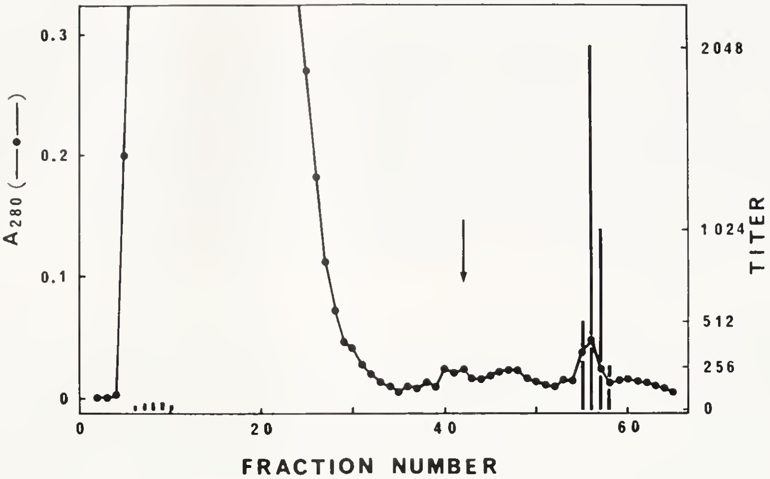

FIGURE 1. Affinity chromatography of *Urechis* lectins. Crude lectin extract obtained by ammonium sulfate precipitation was applied to a lactose-Sepharose 6B column (2.7 × 7.5 cm). The column was washed with medium A until the absorption at 280 nm became negligible and then the eluting medium was changed to 100 m*M* lactose in medium A at the point shown by the arrow. The hemagglutinating activity was eluted at a small peak of A_{280}.

shown in Figure 1. The lectin was purified 1350-fold with the yield of 23% by the procedure as summarized in Table 1.

SDS-PAGE in the presence of 2-mercaptoethanol of the lectin preparation showed two bands corresponding to the molecular weights of 31,000 and 34,000. The two components were isolated by preparative SDS-PAGE (Figs. 2, 3), but they were not separable if the concentration of SDS was less than 0.02%. Both of them retained the hemagglutinating activity (Fig. 2), and were susceptible to thiodigalactoside, lactose, and galactose.

Molecular weights of the lectins

Molecular weights of the lectins were estimated to be 31,000 and 34,000 by SDS-PAGE. However, this difference in the molecular weights was not revealed by other methods. Both lectins showed the same molecular weight of 31,000 by SDS-PAGE in the absence of 2-mercaptoethanol (Fig. 3), 26,000 by gel filtration using Sephadex

TABLE I

Purification of the lectins from the coelomocytes of Urechis unicinctus

Step	Titer	Protein (mg/ml)	Volume (ml)	Sp. act.**	Total activity	Recovery (%)
Extraction	128	3.36	97	38	12,416	100
50–90% AS ppt*	1024	3.92	10	261	10,240	82
Lactose-Sepharose 6B	1024	0.02	2.8	51,200	2,867	23

* Ammonium sulfate precipitation.
** Specific activity; Titer/mg of protein.

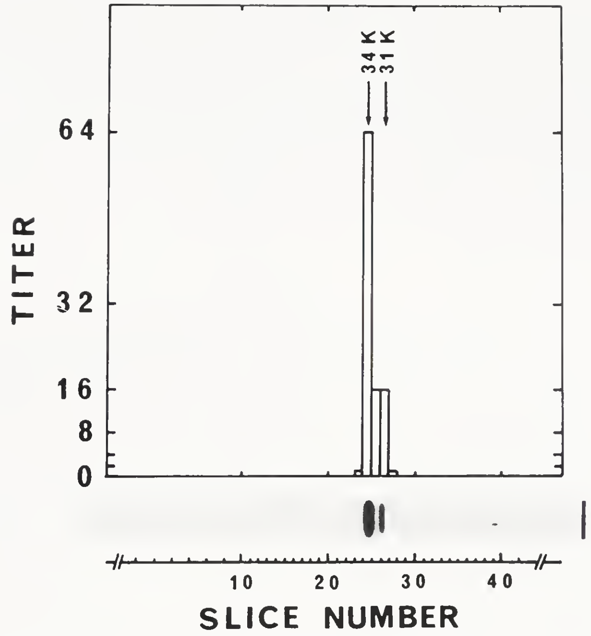

FIGURE 2. Distribution of the hemagglutinating activity after preparative SDS-PAGE. After preparative SDS-PAGE, the gel slice was extracted with medium A overnight at 0°C. The extracts were assayed for the hemagglutinating activity after extensive dialysis.

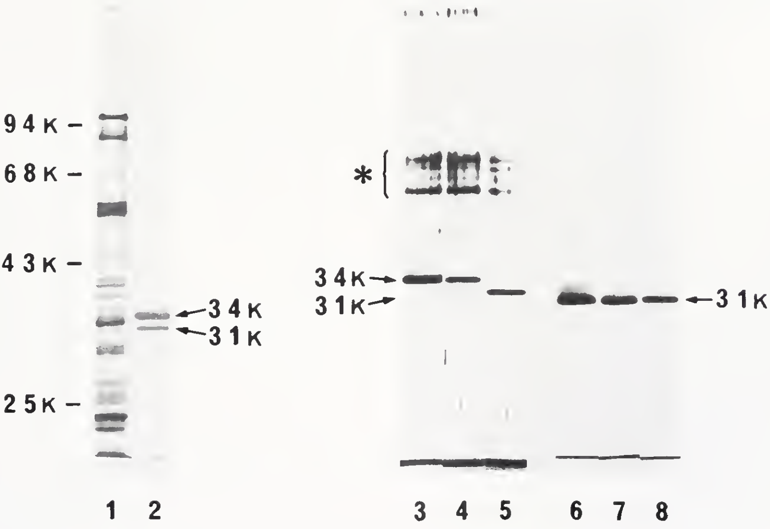

FIGURE 3. Purification of 31,000 and 34,000 lectins by preparative SDS-PAGE. SDS-PAGE (under reduced (*1*–*5*) and unreduced (*6*–*8*) conditions) of lectins. *1.* Flow through fraction of affinity chromatography. *2.* Lectin fraction after affinity chromatography. *3, 6.* The extract of slice No. 25 in Figure 2. *4, 7.* The extract of slice No. 26. *5, 8.* The extract of slice No. 27. Molecular weights of standards are indicated on the left of photographs. Bands indicated by asterisk appeared in the presence of 2-mercaptoethanol presumably by artifact as reported by Tasheva and Dessev (1983).

G-100 in the presence of 100 mM lactose, and 35,000 by gel filtration using Bio-Gel P-100 in the presence or absence of 100 mM lactose (Fig. 4). The elution profiles of the lectins from Sephadex G-100 was not modified by increasing the lactose concentration or by adding 200 mM glucose in the elution buffer. In the absence of lactose, both lectins were eluted much later as a single peak from Sephadex G-100 column. The fractions in the peak of activity from either of the columns were shown by SDS-PAGE to comprise 31,000 and 34,000 lectins.

Characterization of the lectins

Preparations after affinity chromatography were used for general characterization of the lectins because of a limited amount of further purified ones. *Urechis* lectin agglutinated rabbit, human (either type A, B or O), bovine, and swine erythrocytes (Table II). Rabbit erythrocytes were most readily agglutinated by the lectin. The agglutinability was increased by trypsin digestion of erythrocytes, but was not changed by fixation with glutaraldehyde. Therefore, trypsinized and glutaraldehyde-fixed rabbit erythrocytes were used for the standard assays.

Saccharide specificity of the lectins is summarized in Table III. The hemagglutinating activity of the lectins was inhibited by various D-galactosides. Thiodigalactoside was the most potent inhibitor followed by lactose and p-nitrophenyl-β-D-galactopyranoside. Anomeric configuration had a slight effect on inhibition; β-D-galactosides were more effective than α-D-galactosides. Fetuin, having the terminal sialic acids and the penultimate galactose, did not inhibit the lectin activity, while asialofetuin markedly inhibited the activity. A glycoconjugate fraction from the Pronase-digest of coelomocytes also inhibited the lectin. Glucose was a saccharide component only detected in the glycoconjugate by gas-liquid chromatography. Galactose content seemed to be very little, if any (data not shown).

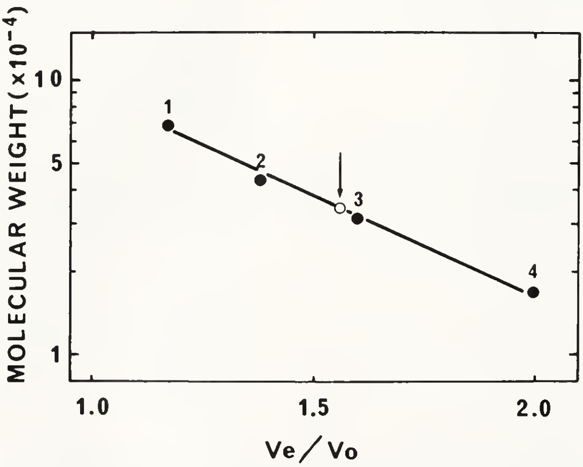

FIGURE 4. Estimation of molecular weight of *Urechis* lectins by a Bio-Gel P-100 column. The active fraction from affinity chromatography was subjected to gel filtration using a Bio-Gel P-100 column (1.3 × 52 cm). The lectins were eluted as a single peak corresponding to the molecular weight of 35,000 either in the absence or presence of 100 mM lactose. Bovine serum albumin (1), ovalbumin (2), deoxyribonuclease I (3), and myoglobin (4) were used as the molecular weight markers.

TABLE II

Erythrocyte specificity of Urechis *lectin*

Erythrocytes		Treatment	Titer
Human	A	none	8
	B	none	4
	O	none	4
Rabbit		none	512
		GA-fixed*	128
		trypsinized**	2048
		trypsinized & GA-fixed	2048
Swine		none	4
		GA-fixed	4
		trypsinized	4
Bovine		GA-fixed	2

The lectin preparation after affinity chromatography was used.
* Glutaraldehyde-fixed erythrocytes.
** Trypsinized erythrocytes.

TABLE III

Saccharide specificity of Urechis *lectin*

Saccharides	Minimal inhibitory concentration (m*M*)
D-Thiodigalactoside	0.05
Lactose	0.10
p-Nitrophenyl-β-D-galactopyranoside	0.10
Methyl-β-D-galactopyranoside	0.20
Methyl-β-D-thiogalactoside	0.39
p-Nitrophenyl-α-D-galactopyranoside	0.78
Methyl-α-D-galactopyranoside	0.78
D-Galactose	0.78
Melibiose	0.78
α-D-Fucose	1.56
L-Arabinose	6.25
N-Acetyl-galactosamine	6.25
Methyl-β-D-xyloside	>50
L-Fucose	>50
D-Glucose	>50

Glycoconjugates	Minimal inhibitory concentration (mg/ml)
Asialofetuin	0.21
Pronase-digest of coelomocytes	0.35*
Fetuin	>3.75
Heparin	>2.5
Glycogen	>8.99

* mg hexose/ml.
Lectin preparation after affinity chromatography was used.
Lectin titer was previously adjusted to 8.

The activity of the lectin was diminished by heating at 60°C for 5 min. No significant loss of the activity was observed after storage in medium A for 3 months at −20°C. No appreciable change in the activity was observed in the absence or presence of 2-mercaptoethanol up to 140 m*M*. However, the activity was preserved much better in the presence than in the absence of 10 m*M* 2-mercaptoethanol at 4°C. The lectins seemed to not require divalent cations for their activity. Most of the activity was lost after several cycles of freezing and thawing. The activity was completely destroyed by proteases (trypsin, Pronase P, α-chymotrypsin, and bacterial protease) but was resistant to glycosidase mixture or alkaline phosphatase. The lectins contained sugars (8.4% of protein). No significant change in the molecular weight was observed even after the treatment of the lectins with glycosidase or alkaline phosphatase. The two lectins were focused by IEF to a single peak of activity with a pI of 5.0–5.5.

DISCUSSION

Most invertebrate lectins have been isolated from hemolymph before or after coelomocyte clumping (Marchalonis and Edelman, 1968; Anderson, 1980; Komano *et al.*, 1980). To prepare hemolymph, little attention has been paid to the integrity of coelomocytes. Thus the source of lectins remains problematical. In *Urechis,* no hemagglutinating activity was detected in the coelomic plasma if the coelomocytes were carefully removed without clumping. Further, a high hemagglutinating activity was detected in the homogenate of washed coelomocytes. From these observations, it is assumed that *Urechis* lectins are localized only in the coelomocytes. Similar distribution of lectin has been reported in lobster (Cornick and Stewart, 1973).

Two lectins were not separable by gel filtration, affinity chromatography, IEF, or PAGE in the absence of SDS or 2-mercaptoethanol. With the exception of molecular size, this may suggest a mutual affinity and/or close similarity in their physicochemical properties. The apparent molecular weight of 34,000 lectin was decreased to 31,000 by SDS-PAGE in the absence of 2-mercaptoethanol. This indicates the presence of disulfide bond(s) in the 34,000 lectin. Since the difference in apparent molecular weights between reduced and unreduced form was rather small, the cystein residues forming disulfide bond(s) seem to locate rather closely along the peptide chain.

The lectins have an affinity to dextran (Sephadex). This affinity seems to be cancelled mostly, but not completely, by the addition of lactose. Lectins may not have an affinity to polyacrylamide gels, since they were eluted from a Bio-Gel P-100 column as a single peak corresponding to the molecular weight of 35,000 in the presence or absence of lactose. The estimated molecular weight was close to those obtained by SDS-PAGE. This suggests that these lectins are monomeric and that the affinity between the lectins is not significant.

Most of the lectin in the coelomocytes was extracted by inclusion of lactose in the extraction medium as reported with chick muscle lectin (Nowak *et al.*, 1977). Only a small portion of the hemagglutinating activity was extractable by lactose-free medium. *Urechis* lectin extracted with lactose-free medium was not adsorbed to the affinity column and was recovered in the break through fractions from a Sephadex G-100 column (data not shown). The lectins seem to bind some macromolecules, which are removed from the lectins by the addition of lactose. One of the candidates for such a putative macromolecule is a glucose-rich glycoconjugate, obtained from coelomocytes after the Pronase digestion. Though the glycoconjugate inhibited the hemagglutinating activity of the lectins, galactose was not detected appreciably in it. Susceptibility of the lectins to the glucose-rich glycoconjugate and their affinity to dextran imply a role for the lectins in the storage of saccharides as suggested by

Sharon and Lis (1972). Ochi (1966) found many glycogen granules in the coelomocytes of *Urechis unicinctus* by histochemical and electron microscopic observations. It is presently uncertain whether the glucose-rich endogenous inhibitor is glycogen. Commercial glycogen and glucose were not inhibitory against the lectins.

We first expected that *Urechis* lectins might participate in the clumping of coelomocytes as a part of self-defense mechanism, but we could not observe any inhibition of the clumping by lactose. Furthermore, the *Urechis* lectins did not agglutinate the glutaraldehyde-fixed coelomocytes which were readily agglutinated by *Ricinus communis* agglutinin. Physiological roles of *Urechis* lectins await further investigation.

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THE EFFECTS OF MULTIPLE INJECTIONS OF BACTERIAL ENDOTOXIN ON BLOOD COAGULATION IN THE TOADFISH, *OPSANUS TAU*

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ABSTRACT

The effects of multiple injections of bacterial endotoxin on blood coagulation in the toadfish, *Opsanus tau,* were studied. Preparations of endotoxin from *Escherichia coli, Aeromonas hydrophila,* or *Vibrio harveyi* (strain 392) were utilized. Three injections of endotoxin were administered at either 6 or 12 h intervals.

Although there was a slight decrease in plasma fibrinogen concentration following the administration of endotoxin, levels did not significantly differ from normal controls. Endotoxins from *E. coli* and *A. hydrophila* initially shortened the prothrombin time (PT), but subsequently the PT returned to normal. In contrast, the partial thromboplastin time (PTT) was not shortened following the administration of bacterial endotoxin. There was no detectable effect on the plasma recalcification time. There were no significant differences between the red and white blood cell levels of control fish and fish that had received endotoxin. Endotoxins from *A. hydrophila* and *V. harveyi* reduced the survival of recipient toadfish, whereas *E. coli* did not increase mortality during the period of observation. Although both control and experimental fish demonstrated increased hematopoietic activity in the kidney, only *O. tau* that had received endotoxin demonstrated marked necrosis of interstitial cells.

Our data indicate that multiple injections of bacterial endotoxin into the toadfish result in alterations of blood coagulation compatible with activation of the blood coagulation mechanism. However, striking evidence of a consumptive coagulopathy, as is observed in mammals following multiple injections of bacterial endotoxin, was not observed. Interestingly, toadfish tolerated doses of endotoxin markedly greater than those sufficient to produce not only alterations of blood coagulation but also rapid death in many mammalian species.

INTRODUCTION

Blood coagulation has been studied in both healthy (Doolittle and Surgenor, 1962; Langdell *et al.,* 1965) and abnormal fish (Katz and Southward, 1950; Bouck and Ball, 1966; Hougie, 1971; Casillas *et al.,* 1975; Casillas and Smith, 1977). Coagulation disorders have been described in post-spawning Pacific salmon (Katz and Southward, 1950; Hougie, 1971) and in Pacific salmon following experimental stress (Bouck and Ball, 1966; Casillas and Smith, 1977). In fish subjected to experimental decompression, coagulation changes similar to those described in mammalian disseminated intravascular coagulation syndromes were observed (Casillas *et al.,* 1980).

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Impaired coagulation also has been observed in fish suffering from gram negative septicemia. Busch (1978) noted that the whole blood clotting time was markedly lengthened in trout infected with *Yersinia ruckeri,* a gram negative bacterium which causes enteric redmouth disease. Similarly, in fish infected with *Aeromonas salmonicida* recalcified plasma clots were more fragile than the normal clots (Field *et al.,* 1944), while blood from eels dying from *Vibrio* sp. infection coagulated poorly during the necropsies (McCarthy, 1976). Interestingly, Umbreit and Tripp (1975) demonstrated a heat stable toxin from *Vibrio anguillarum* which was lethal in goldfish. In addition to bacterial pathogens, prolonged whole blood clotting was observed in salmon infected with the virus responsible for viral hemorrhagic septicemia (Watson *et al.,* 1956).

Others have investigated a wide range of biological effects produced by endotoxin in fish, including lethality (Berczi *et al.,* 1966; Pol and Berg-Blommaert, 1980), antibody response (Paterson and Fryer, 1974; Ingram and Alexander, 1980), pyrogenicity (Reynolds *et al.,* 1977), hepatic glycogenolysis (Wedemeyer and Ross, 1968), and pituitary activation (Wedemeyer, 1969). *In vitro,* endotoxin was inactivated by fish serum (Von Eschen and Rudbach, 1974) and induced consumption of complement in fish serum (Day *et al.,* 1970). In contrast to mammals in which low doses of endotoxin produce pathologic effects, doses greater than 200 mg/kg have been required to produce lethality in fish (Berczi *et al.,* 1966) and intravenous injections as large as 47 mg/kg failed to affect the blood pressure (Wedemeyer and Ross, 1968). However, a lower dose (25 mg/kg) did cause a transient elevation in plasma cortisol six to eight hours post-injection (Wedemeyer, 1969). Following a much higher dose of *V. anguillarum* endotoxin (355 mg/kg), injected intraperitoneally, there was a significant decrease in plasma protein in coho salmon (Harbell *et al.,* 1979).

Although Hougie (1971) postulated that endotoxemia produced disseminated intravascular coagulation in diseased post-spawning salmon and a bacterial toxin was suspected to be active in eels dying from hemorrhagic septicemia due to a *Pseudomonas* species (Andre *et al.,* 1972), there has been no direct evidence linking endotoxin to abnormal blood coagulation in fish, as occurs in mammals (Levin and Beck, 1966). Furthermore, in addition to activating mammalian coagulation systems under certain conditions, multiple injections of endotoxin can produce renal lesions in association with thrombosis and hemorrhage (Bell *et al.,* 1972). Therefore, the following investigation was performed to identify possible effects of multiple injections of endotoxin on the blood coagulation system of fish and to detect related renal lesions. The saltwater teleost, *Opsanus tau,* was the test species. Three different preparations of endotoxin were evaluated: a commercially available lipopolysaccharide derived from *Escherichia coli;* a phenol extract of *Aeromonas hydrophila,* a freshwater fish pathogen; and a distilled water lysate of *Vibrio harveyi* (strain 392), a marine bacterium.

MATERIALS AND METHODS

The toadfish, *Opsanus tau,* was used in all studies. Fish were selected from several hundred *O. tau* that had been captured 2–3 months previously and maintained in large concrete tanks equipped with flow through sea water, at the Marine Biological Laboratory supply department. Fish of approximately the same size (mean weight 168 g ± 6 g (1 SE)), showing normal behavior and no visible external lesions, were chosen for experimentation. The majority of the fish had not been fed since the time of capture and were not fed during the experiments, although in a few later experiments fish were selected from stocks that had had supplemental feeding with *Fundulus heteroclitus* for approximately two months. During the experiments, individual fish were identified by pieces of string inserted through the skin of either the dorsal or pectoral fins and maintained in constantly flowing sea water within 5 gallon plastic buckets.

During either the injection or sampling procedures, the fish were restrained by wrapping them with wet cheesecloth and manually holding them down. To facilitate access to the gill arch vessels, into which intravenous injections were made and from which samples were taken, the two areas of loose skin connecting both the dorsal and ventral edges of the operculum to the body were incised, with no apparent harm to the fish. With the operculum reflected forward, the gill arches were well exposed. Plastic syringes (2.5 ml) and 26G needles were used for all injections or samples.

Preparations of endotoxin

Three types of endotoxin were used: a commercially available lipopolysaccharide prepared from *E. coli,* 026:B6 (Difco Laboratories, Inc., Detroit, Michigan); a phenol extract of *A. hydrophila;* and a crude lysate of *V. harveyi* in distilled water. The *A. hydrophila* (kindly provided by Dr. Alan Scott, Fisheries Department, Auburn University, Auburn, Alabama) was originally isolated from a moribund bluegill, *Lepomis macrochirus,* in a wild population of fish experiencing an epizootic. Phenol extraction of a 36 hour batch culture, grown in Brain Heart Infusion broth (Difco Laboratories, Inc.) at 30°C on a shaker table, was carried out according to the procedure outlined by Staub (1967).

The *V. harveyi* (strain 392), a common marine bacterium, was provided by Dr. Peter Greenberg, Department of Microbiology, Cornell University, Ithaca, New York. *V. harveyi* was grown for 6 hours in a batch culture in complete sea water media (5.0 g yeast extract, 3.0 g peptone, 3.0 g glycerol, 300 ml distilled water, and 700 ml sea water) (Nealson, 1978), at 30°C on a shaker table. After two washings with sterile 3% saline, the cells were boiled for 20 minutes in distilled water and allowed to remain at 4°C for an additional 2.5 hours. Following centrifugation, the supernatant was removed from the pelleted bacterial cells and used as a source of crude endotoxin. Prior to injection, sufficient sodium chloride was added to the distilled water lysate to yield a saline concentration of 0.9%.

Dose of endotoxins

When assayed by the Limulus amebocyte lysate test (Levin and Bang, 1968), the preparations of endotoxin from *A. hydrophila* and *V. harveyi* were equivalent to ≥ 100 mg/ml and 0.1 mg/ml of *E. coli* lipopolysaccharide (Difco Laboratories, Inc.), respectively. The total dose (based on biological activity in the Limulus test) of *V. harveyi* endotoxin varied from 0.004 to 0.008 mg/g of body weight, and of *A. hydrophila* from 2.4 to 4.4 mg/g body weight. A 0.1 mg/ml solution of *E. coli* was used, and the total dose ranged from 0.002 to 0.003 mg/g body weight. Control fish received injections of similar volumes of 0.9% saline.

All injections of *E. coli* and *A. hydrophila* endotoxins and the majority of *V. harveyi* endotoxin injections were administered at 12 hour intervals, designated (0, 12, 24 h). In a few experiments, *V. harveyi* endotoxin was injected at 6 hour intervals, designated (0, 6, 12 h). Control fish were injected using the appropriate schedule.

Blood samples

Since unlimited sampling was not possible due to the fixed number of gill arches, in most cases the initial blood sample was not obtained until after the second injection of endotoxin or saline. The majority of samples were obtained 6 or 12 hours after an injection, depending upon the injection schedule. A control group of fish that had received no injections of either saline or endotoxin also was bled.

The volume of each sample of whole blood was measured in the syringe and after removal of the needle, the blood was expressed into a siliconized glass tube. Sufficient 3.8% sodium citrate was immediately added to produce a citrate:blood ratio of 1:9, the two were thoroughly mixed, and the tube was then placed in wet ice until the total blood cell count was completed. Subsequently, the sample was centrifuged for 10 minutes at 2000 rpm in a table top centrifuge and the plasma then removed from the red blood cells and buffy coat with a pasteur pipet. The plasma sample was either placed in wet ice or held at 4°C in a refrigerator until tests of plasma coagulation could be completed. Although the tests were not affected by storage at 4°C for 48 hours, all determinations were completed within 24 hours after the sample was obtained. Frozen samples were unsuitable and were never used.

Mortality

All mortalities were recorded as hours to death after the initial injection of endotoxin. In several cases, fish were sacrificed *in extremis*. However, some fish died unobserved overnight. In the latter cases, the time of death was estimated as closely as possible, based on the condition of the carcass.

Histopathological analysis

Necropsies were performed on all fish and representative samples of kidney and spleen were removed and fixed with 10% buffered formalin. Following fixation, the tissues were embedded in paraffin; 6 micron sections were prepared and stained with hematoxylin and eosin by standard methods. All tissue sections were examined with light microscopy.

Red and white blood cell counts

The number of both red and white blood cells was determined by a method similar to that described by Klontz and Smith (1968). Briefly, citrated whole blood was diluted 1/200 with Rees-Ecker counting fluid in a red blood cell diluting pipet and allowed to stand for approximately 10 minutes at room temperature. Both chambers of a modified Neubauer hemocytometer were filled from one sample and the counts of the two chambers were averaged. Under 400× magnification, erythrocytes appeared as elliptical cells with an elliptical nucleus and abundant clear to pale blue cytoplasm; while white blood cells were smaller, round, dark blue staining cells with little or no apparent cytoplasm. It was impossible to distinguish thrombocytes from other white blood cells.

Tests of blood coagulation

A small sample (approximately 0.4 ml) of unanticoagulated whole blood was placed in a clean glass tube in order to determine the whole blood clotting time. The tube was gently tilted every 5 to 10 minutes to observe any clot formation. The appearance and time of formation of the clot were noted and the tube was then covered and set aside for later observation of clot retraction.

The fibrinogen concentration in citrated plasma was determined by the serial dilution technique outlined by Hougie (1977). Serial twofold dilutions of plasma (to

1:128) were prepared in 0.14 *M* phosphate buffered saline (PBS), pH 7.2. An equal volume of a solution of bovine thrombin (Sigma Chemical Company, St. Louis, Missouri) containing approximately 1 NIH unit of thrombin in PBS was added to each dilution (final concentration of thrombin in assay, 5 U/ml). Approximately 90–120 minutes after the addition of thrombin, the tubes were checked for the presence of a gel by gently tipping the tube. After the highest dilution containing a gel was recorded, the tubes were covered and allowed to sit at room temperature until the next morning, when the highest dilution containing a gel was recorded again.

Hougie's method for measuring the prothrombin time was used with some slight modifications (Hougie, 1971). Brains of *O. tau,* freed of overlaying meninges and blood vessels, were ground up in PBS. The suspension was placed in a refrigerator at 4°C for 1 hour to allow the largest pieces of brain tissue to settle. The supernatant was then removed and used as a complete thromboplastin, after the dilution of thromboplastin that produced the shortest prothrombin time was determined. Small samples of the complete thromboplastin, twice as concentrated as the optimal dilution, were stored at −20°C and reconstituted with PBS prior to use. The prothrombin time test was performed by mixing equal volumes (0.05 ml) of citrated plasma and complete thromboplastin in a clean glass tube, allowing the mixture to incubate for 30 seconds at room temperature, and then adding 0.05 ml of 0.025 *M* $CaCl_2$. The tube was tilted by hand with a regular motion and the time required for gel formation was measured to the nearest second with a stopwatch.

A partial thromboplastin was extracted from cleaned *O. tau* brains according to the method described by Rapaport *et al.* (1954) and was standardized and stored in the same fashion as the complete thromboplastin. The partial thromboplastin time test was performed according to the method of Goulian and Beck (1965) with only slight modification. The kaolin suspension (10 mg/ml) was added to the partial thromboplastin in the assay tube just prior to the test (0.05 ml of each reagent). Citrated plasma (0.05 ml) was then added to the mixture and allowed to incubate for 30 seconds at room temperature. Following incubation, 0.05 ml of 0.045 *M* $CaCl_2$ was added and the time required for gel formation was measured to nearest second with a stopwatch.

The recalcification time of citrated plasma was measured by mixing equal volumes of undiluted plasma and 0.025 *M* $CaCl_2$ in a clean glass tube. Ordinarily, several plasma samples were assayed simultaneously rather than each sample separately. Therefore, the intervals between observations of one tube were occasionally as long as 30 seconds. Each tube was gently tilted sequentially until a gel was detected. For all coagulation tests, the entire procedures were performed at room temperature (25°).

All data were analyzed by the Student *t*-test.

RESULTS

Survival after endotoxin administration

Injection of *V. harveyi* endotoxin (0, 12, 24 h schedule) caused a significant decrease ($P < 0.05$) in the mean survival time in comparison with saline injected fish (Fig. 1). Furthermore, *V. harveyi* (0, 6, 12 h schedule) significantly decreased the mean survival time in comparison with the other injection schedule of *V. harveyi* (0, 12, 24 h) ($P < 0.05$). Although differences in survival between the different types of endotoxin were not significant, *V. harveyi* and *A. hydrophila* endotoxins appeared more lethal than did *E. coli* endotoxin.

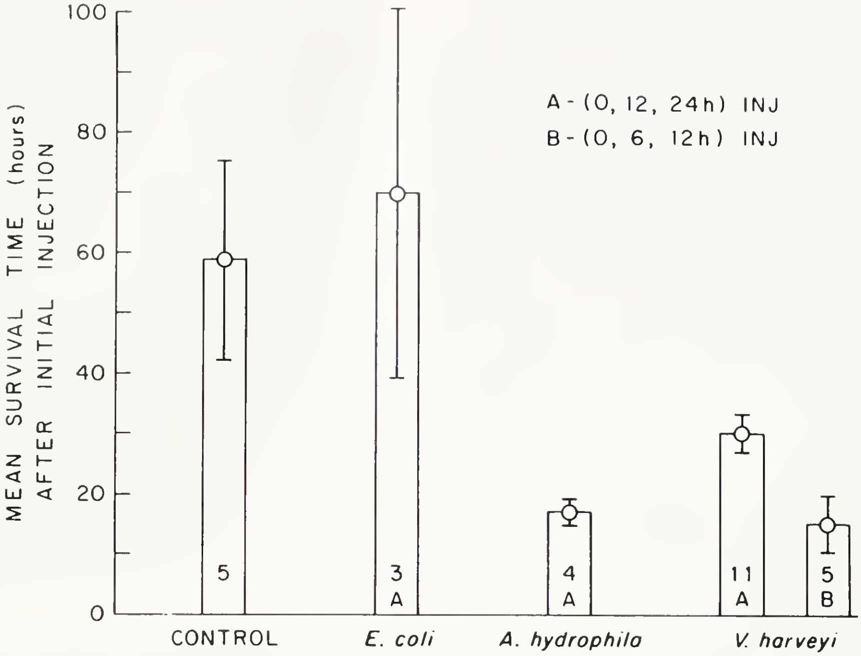


FIGURE 1. *Survival after endotoxin administration.* Bars indicate the mean survival time ± 1 standard error of the mean (S.E.M.) following the initial injection of endotoxin. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. The type of endotoxin is designated beneath each bar.

Plasma fibrinogen concentration

The mean plasma fibrinogen titer, *i.e.,* the highest dilution of plasma that formed a detectable gel after addition of thrombin, of normal unbled *O. tau* was 1:32 (Fig. 2). After three injections of 0.9% NaCl (approximately 10 ml total dose) and removal of a total of approximately 4.0 ml of blood in two earlier blood samples, the fibrinogen concentration fell only insignificantly (Fig. 2). A decrease after repeated injections and removal of samples also occurred in animals injected with endotoxin, although the reductions of fibrinogen in endotoxin injected fish were not significantly lower than corresponding control fish. However, the fibrinogen concentration after only one injection of *E. coli* endotoxin was significantly greater ($P < 0.01$) than in uninjected control fish. Subsequently, in the fish injected with *E. coli* endotoxin, the concentration of fibrinogen in the post-third injection sample was significantly decreased ($P < 0.05$) from the post-first injection sample (Fig. 2).

Prothrombin time

The prothrombin time (PT) of uninjected control fish was approximately 33 seconds and shortened slightly after animals had received three injections of 0.9% NaCl and two blood samples had been taken (Fig. 3). In comparison with saline injected animals, one injection of *A. hydrophila* or *E. coli* endotoxin caused the PT to shorten significantly ($P < 0.01$) or insignificantly, respectively. Following two more

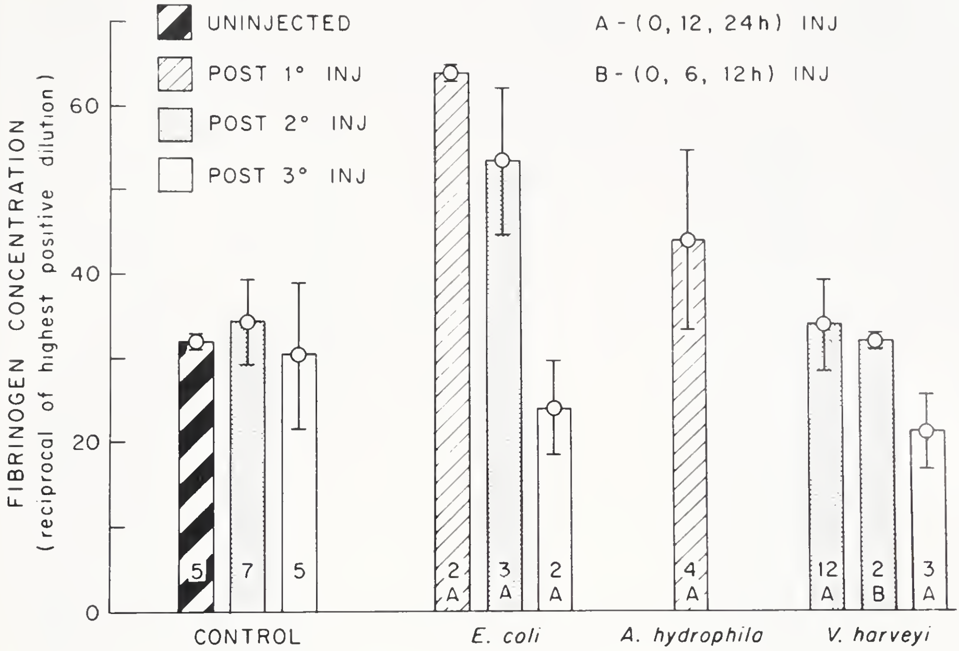


FIGURE 2. *Plasma fibrinogen concentration.* Bars indicate the reciprocal ± 1 S.E.M. of the highest plasma dilution that formed a visible gel following the addition of bovine thrombin (final concentration, 5 U/ml). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Lightly striped bar = post-first injection. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars. Initially uninjected controls were used for statistical comparison with post-first injection samples from experimental animals to minimize the number of samples required from saline injected control animals. Additional samples were obtained from controls after the second and third injections of saline.

injections of *E. coli* endotoxin, the PT lengthened to comparable control levels. *V. harveyi* endotoxin did not significantly change the PT in comparison with saline injected animals, nor was there a significant difference between injection schedules.

Interestingly, in preliminary experiments, complete thromboplastin (brain extract in 0.9% NaCl) prepared from the red hake, *Urophycis chuss,* also a marine fish, resulted in a longer PT than did the homologous thromboplastin prepared from *O. tau* brains.

Partial thromboplastin time

The partial thromboplastin time (PTT) of normal control animals was approximately 55 seconds, and in contrast to the PT, became prolonged, though not significantly, following multiple injections of saline and removal of blood samples (Fig. 4). No significant differences were observed between endotoxin and saline injected animals. In *E. coli* endotoxin injected animals, there was an increase in PTT between the post-second injection sample and the post-third injection sample, although the increase was not significant.

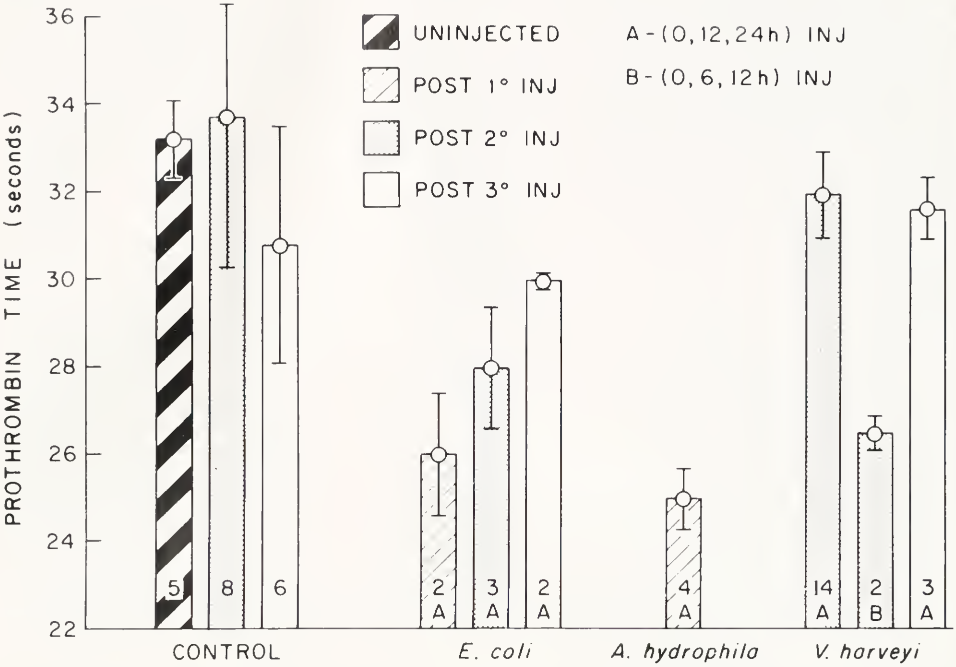


FIGURE 3. *Prothrombin time.* Bars indicate the mean time in seconds ± 1 S.E.M. for gel formation to occur following the addition of Ca^{+2} to an equivolume mixture of plasma and saline brain extract (See Methods). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Lightly striped bar = post-first injection. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Whole blood coagulation time

Due to procedural difficulties, it was impossible to obtain and observe sufficient numbers of samples for quantitative and statistical analysis. However, there was a clear trend in both experimental and control animals towards a shortening of the whole blood coagulation time (WBCT) following the initial blood sample. Although in all groups of fish the WBCT in the first sample was highly variable and frequently greater than 2 hours, (mean, 2.3 ± 0.5 h), the WBCT in the second sample was in all cases less than 40 minutes and in two thirds of the second samples the blood coagulated within 20 minutes. In no animals did the WBCT lengthen with repeated sampling.

In addition to changes in the coagulation time, the appearance of the clot also altered. When the coagulation time in the first sample was lengthened, *i.e.,* hours rather than minutes elapsed until coagulation, the clot that eventually formed was only semi-solid and did not involve the entire volume of the sample. However, in later samples when the coagulation time shortened, the clot became more solid and usually involved the entire sample. Due to the small size of the sample and the poor quality of the clots, it was often impossible to evaluate the degree of clot retraction that had occurred. However, clot retraction was observed in those samples in which solid clots formed initially.

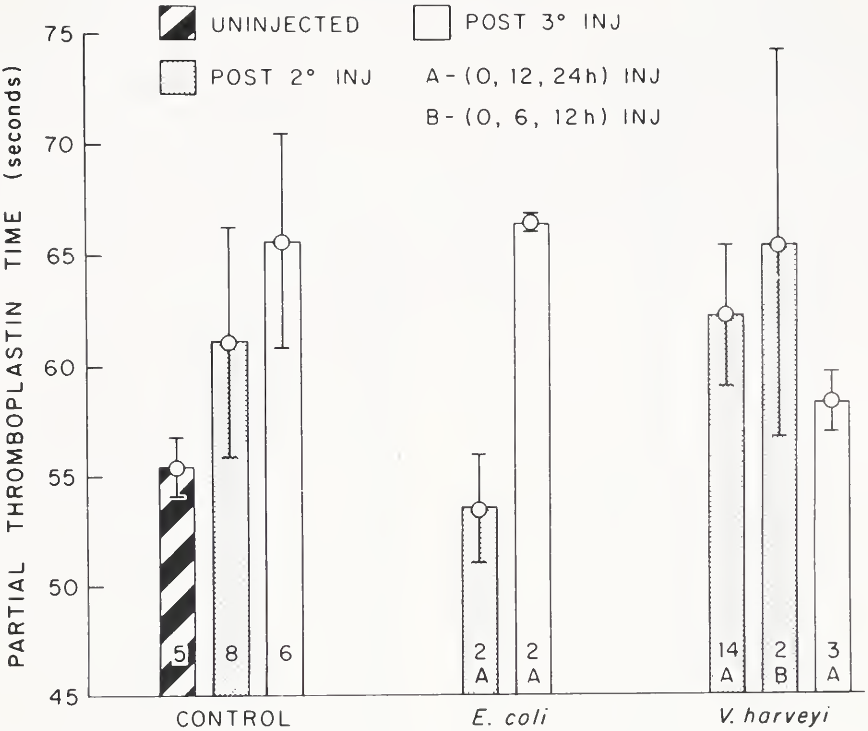


FIGURE 4. *Partial thromboplastin time.* Bars indicate the mean time in seconds ± 1 S.E.M. for gel formation to occur following addition of Ca^{+2} to an equivolume mixture of plasma, partial thromboplastin, and kaolin suspension (See Methods). The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h) and B = (0, 6, 12 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Plasma recalcification time

The plasma recalcification time was variable in both normal and experimental animals with a range of 1 to 8 minutes; and the endpoint was not well defined because coagulation generally occurred during a 30 to 60 second period. Therefore, the recalcification time was difficult to evaluate accurately. All samples did eventually form a gel, with the exception of one uninjected control that failed to clot even after 15 minutes of observation. From the available observations there appeared to be no effect of endotoxin on the recalcification times.

Red blood cell count

The red blood cell count declined significantly ($P < 0.01$) from a normal level in uninjected fish of 4.6×10^5 erythrocytes/mm^3 to approximately 1.4×10^5 erythrocytes/mm^3 following three injections of saline and two blood samples (Fig. 5). Similarly, animals injected with *V. harveyi* (0, 12, 24 h) showed a significant decline ($P < 0.05$) in the number of red blood cells between the post-second injection and post-third injection samples. There were, however, no significant differences between any of the endotoxin and comparable saline injected animals.

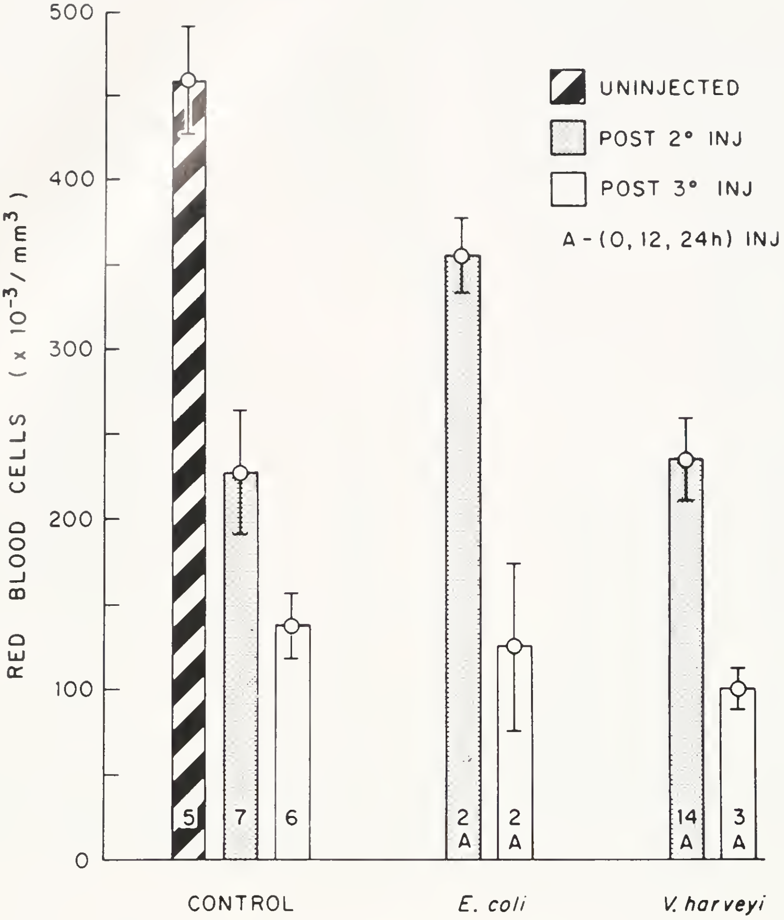


FIGURE 5. *Red blood cell count.* Bars indicate the mean number ± 1 S.E.M. of erythrocytes/mm^3. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

White blood cell count

As in the red blood cell counts, there was a general decline in white blood cell numbers in the post-third injection samples for all treatments (Fig. 6). However, this decline was not significant in contrast to the decreases observed in red blood cell numbers.

The number of white blood cells was significantly less ($P < 0.01$) in the *V. harveyi* endotoxin (0, 12, 24 h) injected animals than in saline injected animals, in the post-second injection samples (Fig. 6). By the post-third injection samples, there was no difference between saline and *V harveyi* injected animals, although the white blood cell count in the *V. harveyi* injected animals had continued to decline.

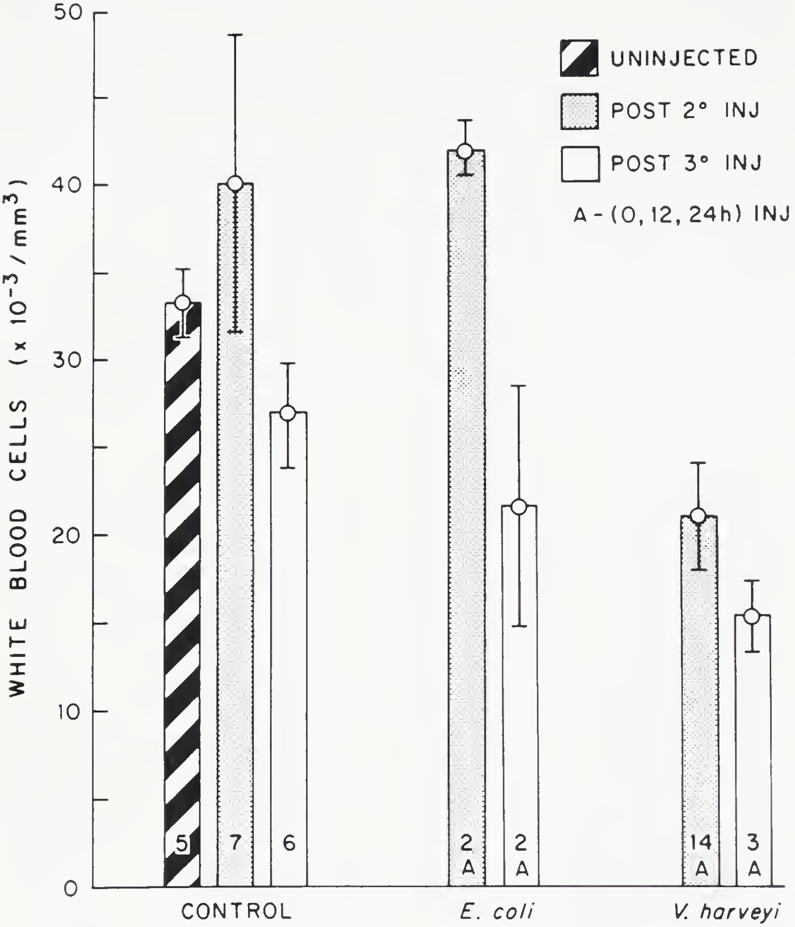
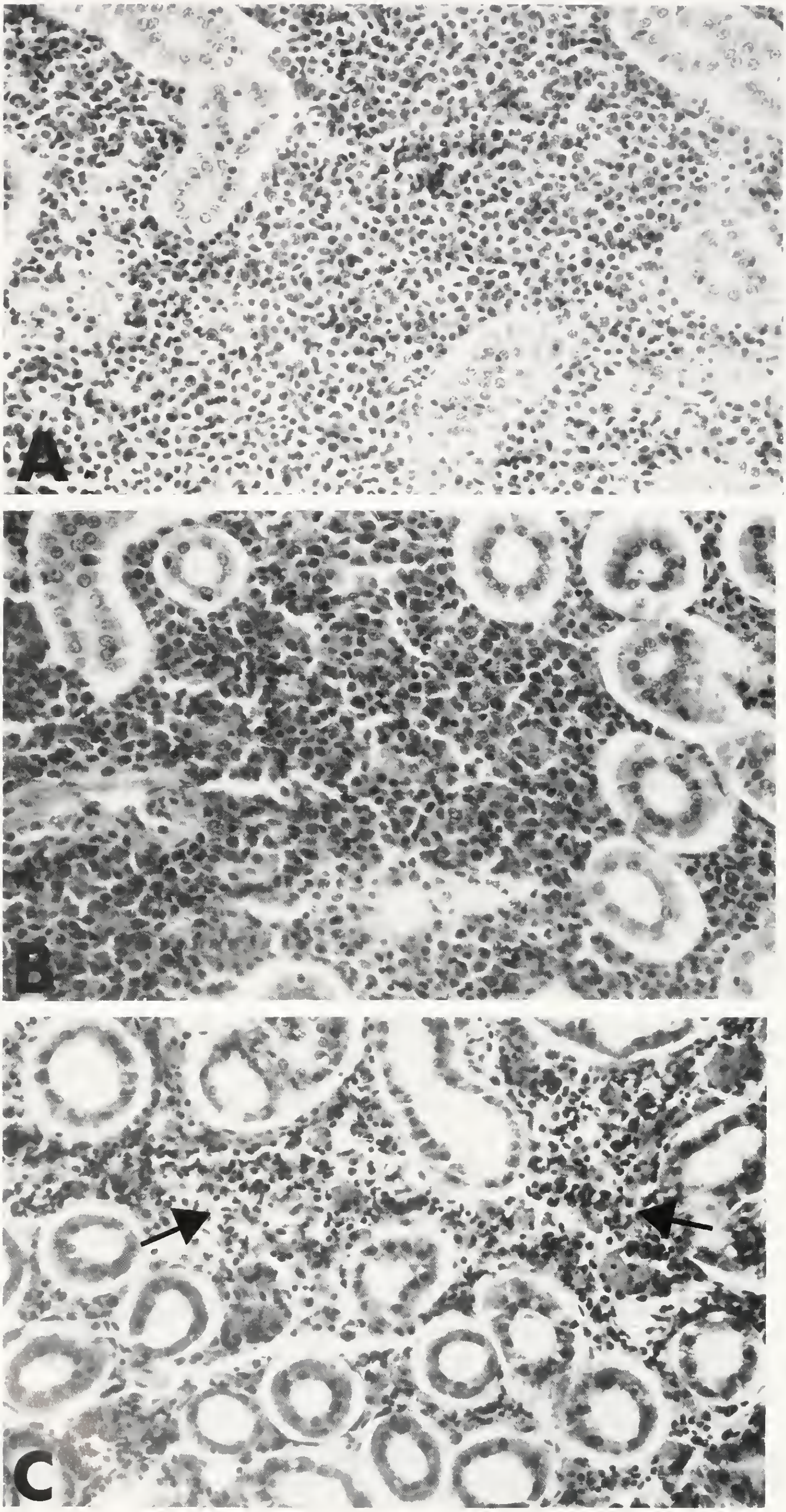


FIGURE 6. *White blood cell count.* Bars indicate the mean number ± 1 S.E.M. of white blood cells/mm^3. The control data from both of the injection schedules (0, 12, 24 h and 0, 6, 12 h) did not differ significantly and therefore were combined. Endotoxin injection schedules: A = (0, 12, 24 h). Number of fish in each sample is indicated in each bar. Heavily striped bar = uninjected controls. Stipled bar = post-second injection. Open bar = post-third injection. The type of endotoxin is indicated beneath each cluster of bars.

Clinical observations

On several occasions, it was impossible to obtain a blood sample from an animal due to apparent stasis of blood within the branchial vessel, as evidenced by a lack of hemorrhaging following withdrawal of the needle. Prior to these unsuccessful sampling attempts, the fish were unresponsive to stimuli, and succumbed shortly after being returned to water.

After injections of either endotoxin or saline, many fish demonstrated a period of abnormal behavior, which lasted up to 30 minutes and was characterized by alternating periods of frenzied swimming and extreme lethargy. During the latter


A
B
C

part of the injection schedule, the periods of lethargy were more pronounced and lengthened. Furthermore, in many instances a transient change from a normal dark brown color to a pale yellowish-brown color accompanied this period of abnormal behavior.

Histological examination

The primary change observed in the kidneys was a loss of normal interstitial architecture due to a combination of cellular necrosis and compensatory hematopoietic activity. Generally, kidneys of saline injected fish displayed hematopoietic activity in the absence of cellular necrosis, whereas the kidneys of endotoxin injected fish, paticularly those injected with *V. harveyi* endotoxin, demonstrated marked cellular necrosis in addition to compensatory hematopoietic activity (Fig. 7). The renal interstitial tissue of an uninjected fish (Fig. 7a) or a saline injected fish (Fig. 7b) appears intact and contains a normal amount of hematopoietic cells. However, following injection of *V. harveyi* lysate, there was a marked loss of hematopoietic tissue due to severe necrosis (Fig. 7c). Although in this fish the necrosis was severe, other fish injected with endotoxin occasionally showed similar, but less marked lesions. Tubular necrosis was also occasionally observed. Since *O. tau* is aglomerular, it was impossible to observe glomerular fibrin deposition as is observed in the generalized Shwartzman reaction. No histological differences between groups were noted in sections of spleens.

DISCUSSION

Blood coagulation was evaluated in the toadfish, *Opsanus tau,* following multiple injections of endotoxin, and although differences between saline and endotoxin injected animals were not necessarily consistent with a consumption coagulopathy in all cases, there were several significant changes related to the injection of endotoxin. The most obvious effect of repeated injections and removal of blood samples was the development of anemia in all fish, both experimental and control. Assuming that a blood volume of approximately 3% of body weight for marine fish, as reported by Thorson (1961), applies to *O. tau,* each sample of 1.5 to 2.0 ml removed up to 30% of the total blood volume. This large blood loss was undoubtedly a significant stress and accounts for the observed anemia; and furthermore must be considered when other hematological and histopathological changes are interpreted.

Although high doses of endotoxin caused increased mortality, there was no correlation between lethality and dose of endotoxin as measured by the Limulus amebocyte lysate assay. *V. harveyi* endotoxin was more lethal than approximately the same total dose of *E. coli* endotoxin. Furthermore, *A. hydrophila* endotoxin, which was injected in much greater amounts ranging from 2.4 to 4.4 mg/g, was no more lethal than *V. harveyi* endotoxin. Lack of correlation between the dose and lethality may have resulted in part from the different sources of endotoxin and their manner of preparation. In any case, the amount of endotoxin injected was far in excess of the dose required to perturb mammalian systems. However, we are unaware of any report in which endotoxin levels were measured in the blood of fish during gram negative septicemia,

FIGURE 7. *Photomicrographs of posterior kidney.* Normal interstitial hematopoietic tissue in the posterior kidney of uninjected fish (7A) and fish injected three times with saline (7B) is compared with abnormal posterior kidney of fish injected two times with *V. harveyi* lysate (7C). Note the areas of necrosis and loss of interstitial cellularity (indicated by arrows) in fish after injection of *V. harveyi* lysate (7C). (Hematoxylin-eosin stain; original magnification ×100).

although a severe bacteremia of 10^6 viable bacteria/ml of blood has been reported in eels experimentally infected with *Aeromonas liquefaciens* (Hatai, 1972).

The clinically observed period of abnormal behavior following injections represents a non-specific stress response. Pigmentation changes also often occur in response to general stress and are frequently detected during bacterial infections (Richards and Roberts, 1978).

The observation of blood stasis in the branchial vessels of moribund fish may be in part explained by hypercoagulability of the blood. When a sample was drawn from such an animal, the blood invariably coagulated within a minute. Hypercoagulability may have been due to local tissue damage following multiple samples and injections in addition to the direct effects of bacterial endotoxin. The removal of a substantial volume of blood also probably caused decreased blood pressure which further contributed to the stasis of blood.

At the post-second injection sample, *V. harveyi* endotoxin injected animals had significantly lower leukocyte counts than comparable saline injected animals. It is tempting to postulate that the decrease in leukocytes is analogous to the initial decrease in white blood cell levels observed in mammals injected with endotoxin, although in mammals leukopenia occurs much sooner after injection of endotoxin. However, the ultimate decline in the leukocyte number in the final samples obtained from all fish was probably produced, at least in part, by the same factors that caused the aforementioned anemia. Interestingly, the reduction in leukocytes was not nearly as severe as the decline in erythrocytes.

Inasmuch as the injection and sampling schedule caused a decline in the number of circulating blood cells, one might also have observed a lengthening of the plasma coagulation tests due to the physical removal of essential coagulation factors. In some tests, however, there was a shortening rather than a lengthening of the coagulation times. The whole blood coagulation time (WBCT) clearly showed this trend. Not only did the clot form more quickly in later samples, but the quality of the clot was better, indicating that if there was a diminution of some clotting factors, it did not affect the rate of coagulation.

The normal fibrinogen titer of *O. tau* was within the normal ranges of human (Hougie, 1977) and salmonid (Casillas *et al.*, 1975) fibrinogen concentrations. The insignificant decreases in fibrinogen levels in the post-third injection samples in both saline and *V. harveyi* endotoxin injected animals may have resulted from repeated sampling. However, as indicated by the WBCT and plasma coagulation tests, there was still a sufficient concentration of fibrinogen available for clot formation. There is no clear explanation for the observed elevated fibrinogen level following the initial injection of *E. coli* endotoxin, though it may represent early hypercoagulability. In mammals, elevated fibrinogen levels have been observed in both clinical states (Sack *et al.*, 1977) and experimental models (Cooper *et al.*, 1971) associated with disseminated intravascular coagulation.

The prothrombin time (PT) measured in *O. tau* was markedly longer than values reported for salmonids (Hougie, 1971; Casillas *et al.*, 1975). In comparison to salmon, *O. tau* is extremely sluggish and may not require a rapidly responsive coagulation system. Additionally, the PT appeared unresponsive to repeated sampling and injections, since the control times remained fairly constant in all samples (there was an insignificant shortening in the post-third injection sample). It is noteworthy that in preliminary PT tests, the use of a saline brain extract from the red hake, *Urophycis chuss*, rather than the extract from *O. tau*, resulted in markedly lengthened PT times. While the effects of different thromboplastins have been studied with fish, comparisons were made between brain tissues from different classes of animals (Doolittle, 1962; Hougie, 1971). However, our preliminary results indicate that there appear to be differences in fish thromboplastins obtained from separate species.

Although the PT has been reported to be prolonged in consumptive coagulopathy syndromes (Colman *et al.*, 1972), prolongation has not been uniformly detected. However, both *E. coli* and *A. hydrophila* endotoxins appeared to affect the coagulation system of *O. tau*, causing a shortening of the PT after the initial injection. In *E. coli* endotoxin, this initial shortening was followed by a steady lengthening of the PT, although it never became elevated above control values.

The effect of *V. harveyi* endotoxin on the PT is difficult to explain. While two injections of the endotoxin administered at 12 hour intervals had no apparent effect on the PT, two injections given at 6 hour intervals caused an apparent decrease in the PT. Possibly, the two injections of *V. harveyi* endotoxin at 6 hour intervals were close enough together to produce a level of endotoxemia sufficient to cause a hypercoagulable state similar to that observed after one injection of *E. coli* or *A. hydrophila*. In contrast, injections at 12 hour intervals may have allowed sufficient time for the endotoxin to be cleared from the circulation after the first injection.

When compared with values reported in salmonids (Hougie, 1971; Casillas *et al.*, 1975), the partial thromboplastin time (PTT) in *O. tau* was longer, though to a lesser extent than that observed in the PT. Unlike the PT, the PTT increased in both saline and endotoxin injected animals, following multiple injections and samples. Whether these two different responses to the same stimuli by the coagulation system reflect physiologically significant differences between extrinsic and intrinsic coagulation systems in *O. tau* is unclear. Our data suggest that both systems are present and that repeated sampling can more easily deplete some factor(s) involved with intrinsic coagulation in *O. tau*.

The recalcification time (Recal time) of *O. tau* was longer than the Recal time of the tautog, *Tautoga onitis*, a saltwater fish (Doolittle and Surgenor, 1962), and failed to demonstrate a good endpoint. The Recal time, like the WBCT, is dependent on the plasma concentrations of many coagulation factors and therefore is a good indicator of the overall function of the coagulation system. However, unlike the WBCT, which showed dramatic decreases particularly just prior to death, there appeared to be no comparable shortening of the Recal time. A partial explanation for this difference may be that activated thrombocytes were present in the whole blood but were absent from the samples of plasma. The contribution of thrombocytes to fish coagulation has been reported previously (Katz and Southward, 1950; Doolittle and Surgenor, 1962).

The results of histological examinations are somewhat difficult to interpret. Since the interstitial tissue of fish is the primary hematopoietic organ the lesions in the interstitial tissue could not be definitely linked with the injection of endotoxin. Due to the experimentally induced anemia, histological changes characteristic of compensatory hematopoietic activity, unrelated to the endotoxin injections, occurred to some degree in all fish. Nevertheless, injections of endotoxin appeared to cause more severe lesions, characterized by cellular necrosis in the interstitial tissue. Since endotoxin injected animals demonstrated increased mortality, these lesions are likely to be pathological.

As noted earlier, *O. tau* is a particularly sluggish fish and this may account, in part, for the lack of response of its blood coagulation system to endotoxin. In recent studies of rainbow trout, a highly active fish, changes in the coagulation system similar to disseminated intravascular coagulation were measured during experimental *Y.*

ruckeri infections (Miller, 1982; Miller, 1983). Furthermore, preliminary evidence indicated that intracardial injection of *Y. ruckeri* endotoxin caused a similar condition. These marked differences between species of fish warrant further consideration.

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DENSITY IS ALTERED IN HYDROMEDUSAE AND CTENOPHORES IN RESPONSE TO CHANGES IN SALINITY

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ABSTRACT

Laboratory experiments have determined the behavioral and gross physiological responses of hydromedusae and ctenophores subjected to sudden changes of salinities in the range that might be encountered in nature. Nine species of hydromedusae (*Aequorea victoria, Aglantha digitale, Bougainvillia principis, Gonionemus vertens, Phialidium gregarium, Polyorchis penicillatus, Proboscidactyla flavicirrata, Sarsia tubulosa, Stomotoca atra*) and two species of ctenophores (*Bolinopsis infundibulum, Pleurobrachia bachei*) were transferred from natural sea water of 30.5‰ to modified sea waters of 19–38‰. Most species altered their density within a few hours by osmoconforming to salinities ranging from 23–38‰, so that equilibrium buoyancy (either positive, neutral, or negative, according to species) was regained along with normal behavior. Even salinity differences of only 1–2‰ required 30–60 minutes adjustment time. Prior to regaining their equilibrium buoyancies, differences in relative density caused medusae and ctenophores to sink when introduced to low salinity water and to float in high salinity water. Hence, simple density differences combined with the natural intermittent swimming behavior of hydromedusae suggest that in many cases medusae may not actually be able to cross sudden density gradients. In the event that a medusa or ctenophore is moved into water of a different salinity, however, its ability to adjust will allow the animal to resume normal swimming and feeding activities within a short time.

INTRODUCTION

Medusae and ctenophores typically drift or swim weakly in the plankton. Each species has a characteristic inherent buoyancy which causes it to float, sink, or hover when at rest (Mills, 1981b). This species-specific "steady state" buoyancy (which will henceforth be referred to as equilibrium buoyancy) is presumably determined by regulation of sulphate ion within the mesogloea in combination with body construction (Mills and Vogt, 1984). The property of floating, sinking, or hovering is closely coupled with species-specific swimming and feeding behaviors of jellyfish in nature.

Most species of medusae and ctenophores have lifespans varying between several days and several months. Many species occur within rather narrow depth limits in the water column, although these depths may be modified by diel vertical migrations of up to several hundred meters (Benović, 1973; Moreira, 1973; Mills, 1982). If it is correct to assume that most jellyfish travel many miles in their lifetimes, then in many cases they will encounter waters of different salinities in their wanderings. It is of interest to determine whether these organisms are able to adapt to the range of

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salinities which might be encountered in nature, or, conversely, whether variations in salinity provide a hazard to which many planktonic medusae succumb.

Recently Leonard (1980) observed that the hydromedusa *Sarsia tubulosa* is able to vary its density in order to remain positively buoyant in salinities varying from 20 to 27‰. She did not, however, distinguish between active ionic regulation of buoyancy and passive change in density due to osmotic accommodation. Mills and Vogt (1984) determined that active sulphate exclusion is an important factor in buoyancy regulation, but that ion concentrations do not vary over time in a way that might facilitate diel vertical migration of medusae and ctenophores. In the present paper, the general phenomenon of density regulation in response to changes in salinity is investigated. Several species of medusae and ctenophores with different equilibrium buoyancies have been exposed to a variety of salinities in order to determine (1) whether they can regulate their density in response to salinity changes and (2) the rate of any such adjustments.

MATERIALS AND METHODS

Field conditions

In the nearshore coastal and inland marine waters of Washington state (U. S. A.) and British Columbia (Canada), at least 60 species of hydromedusae and 11 species of ctenophores have been collected (Arai and Brinckmann-Voss, 1980; Mills, 1981a). These jellyfishes comprise a substantial component of the zooplankton from April through October; a few of the species are present year-round. The majority of species are characteristic of surface waters (Mills, 1982).

Salinity of these inland waters (which include Puget Sound, the San Juan Archipelago, and the Strait of Georgia) is usually between 28 and 31‰ although salinities from 24–35‰ are common; surface waters in some areas may drop below 15‰ during times of heavy rainfall or snowmelt (Collias and Barnes, 1966; Herlinveaux and Giovando, 1969; U. S. Dept. of Commerce, 1974). Water temperature may range from about 5° to 18°C, but in many areas the annual fluctuation is only a few degrees, temperatures averaging about 11°C.

According to Herlinveaux and colleagues (1961, 1962, 1969), a pycnocline exists throughout the year in most of these areas, with water of lower salinity near the surface. Salinity changes are more important than temperature changes in forming the pycnocline. In summer, the temperature gradient reinforces a density structure predetermined by the salinity gradient, and in winter when there is no thermal gradient, the pycnocline occurs at the halocline. The greatest drop in density occurs between 0 and 25 m; below 50 m only a small density gradient occurs year-round.

Animals

Nine species of hydromedusae (*Aequorea victoria, Aglantha digitale, Bougainvillia principis, Gonionemus vertens, Phialidium gregarium, Polyorchis penicillatus, Proboscidactyla flavicirrata, Sarsia tubulosa, Stomotoca atra*) and two species of ctenophores (*Bolinopsis infundibulum, Pleurobrachia bachei*) were collected in glass or plastic beakers from surface waters near the Friday Harbor Laboratories in the San Juan Archipelago, Washington. *Gonionemus* medusae were collected in Mitchell Bay, San Juan Island, and *Polyorchis* medusae were collected in Shoal Bay, Lopez Island. All other species were collected from beside the floating docks of the Friday Harbor Laboratories. Only animals which appeared to be in excellent condition were used for the experiments.

Modified sea waters

Within a few hours of collection, the medusae or ctenophores were transferred to modified sea water in order to determine behavioral and gross physiological responses to sudden changes in salinity. The experiments were conducted in 1000 or 500 ml glass beakers maintained at ambient sea water temperature (11–14°C) by immersion in an indoor running sea water table. The contents of the beakers ranged in salinity from approximately 19‰ to 38‰ as follows: (1) 60% sea water, 40% fresh (tap) water (19‰ ± 1.0‰), (2) 75% sea water, 25% fresh water (23.0‰ ± 1.0‰), (3) 90% sea water, 10% fresh water (27.5‰ ± 1.0‰), (4) 100% sea water (30.5‰ ± 1.0‰), (5) 100% sea water plus 8 g NaCl per liter (38.0‰ ± 1.0‰). Additionally, salinities 1‰ and 2‰ below and 1‰ above ambient sea water (which will henceforth be referred to as "normal" sea water) were used with *Aequorea* and *Phialidium.* Salinities were measured with an Endeco Type 102 handheld Refractometer/Salinometer.

Depending on sizes of medusae or ctenophores, 2–12 animals of the same species were placed in a beaker together. Several sets of similar experiments were made with each species during the summer months of 1979–1982 as follows: *Aequorea victoria* (8 sets, 146 medusae total); *Aglantha digitale* (5 sets, 62 medusae total); *Bougainvillia principis* (2 sets, 60 medusae total); *Gonionemus vertens* (4 sets, 100 medusae total); *Phialidium gregarium* (6 sets, 207 medusae total); *Polyorchis penicillatus* (3 sets, 61 medusae total); *Proboscidactyla flavicirrata* (5 sets, 68 medusae total); *Sarsia tubulosa* (6 sets, 192 medusae total); *Stomotoca atra* (4 sets, 128 medusae total); *Bolinopsis infundibulum* (3 sets, 59 animals total); and *Pleurobrachia bachei* (2 sets, 44 animals total). (In each set of experiments, all salinities were used, but the numbers of animals were not always equal, subject to availability.)

The medusae and ctenophores were observed for several hours after they had been transferred to new salinities, until their buoyancy and activities returned to normal or until it was obvious that such an adjustment would not be accomplished. In many cases, the animals remained in the new salinities for as long as 48 hours, after which they were returned to natural sea water (approximately 30‰). Their reactions and ability to adjust to this second abrupt salinity change were also observed.

Mesogloeal osmolarity

Because of its relatively large size, *Aequorea victoria* was chosen for measurements of the colligative properties of jellyfish mesogloea in different salinities. Osmolalities of the various dilutions of sea water and of the mesogloea of *Aequorea* were measured in order to determine whether osmotic accommodation was responsible for the change in jellyfish density that followed exposure to water of a new salinity. *Aequorea* medusae from the field were placed in 75%, 90%, 97%, 100%, and 127% sea water for 24 hours during which they adjusted to the change, regaining their normal buoyancies relative to the surrounding media. Samples of mesogloea were obtained by pushing a cork borer through the thickest portion of an *Aequorea,* where the mesogloea was about 15–20 mm thick. The exumbrellar and subumbrellar epithelia were trimmed away and the mesogloeal core was then homogenized in a Pyrex tissue grinder. Freezing points of 2 ml samples of sea water or of homogenized mesogloea were measured with a Fiske Osmometer.

Isotonic Percoll experiments

Four species of medusae (*Aequorea victoria*—10 animals, *Phialidium gregarium*—26 animals, *Proboscidactyla flavicirrata*—8 animals, *Sarsia tubulosa*—10 animals) were placed in a mixture of sea water and Percoll (Pharmacia Fine Chemicals) which changed the density, but not the tonicity of sea water. Percoll is a colloidal suspension of silica coated with polyvinyl pyrollidone; it is nontoxic, and exhibits low osmolality, low viscosity, and high density. After preparing dialysis tubing (Brewer *et al.*, 1974), 100 ml of Percoll was dialyzed against 5 changes of 3 liters of sea water over 22 hours. The density of Percoll after dialysis was 1.148 g/ml. The density of normal sea water with salinity 31.0‰ was 1.026 g/ml and density of hypertonic sea water made by adding 8 g NaCl per liter for a final salinity of 38.5‰ was 1.034 g/ml. One part dialyzed Percoll was added to 14 parts sea water for a final density of 1.034 g/ml (all measurements at 13–15°C). Osmolalities of the above mixtures were measured on a Fiske Osmometer and found to be 800 mOsm/kg for 31.0‰ sea water, 785 mOsm/kg for Percoll-sea water (1:14 v/v) and 1146 mOsm/kg for 38.5‰ sea water.

Behavior and relative buoyancies of medusae in the Percoll-sea water mixture were observed for 24 hours. The medusae were then returned to normal sea water in order to check for any subtle adjustments in density (and relative buoyancy) that may have occurred in the Percoll-sea water mixture.

RESULTS

A standard pattern of events occurred when any species of medusa or ctenophore was introduced to water of a different salinity (Fig. 1). When placed in hypotonic sea water, the jellyfish were relatively dense. Those individuals that had been negatively buoyant or neutrally buoyant at equilibrium in normal sea water sank to the bottom of the experimental beakers; positively buoyant species sank if the drop in salinity

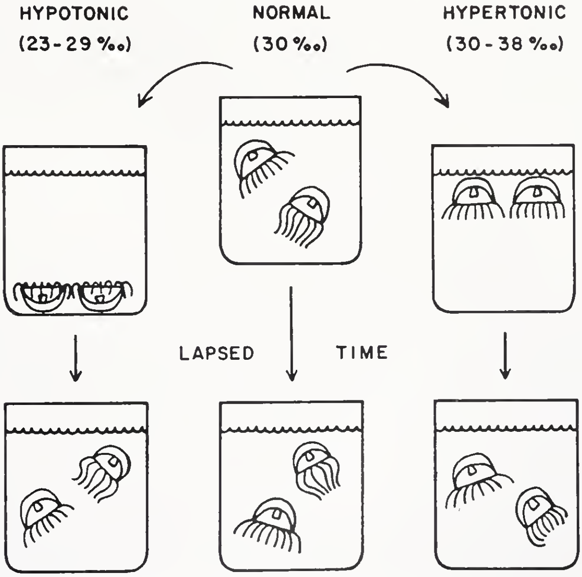

FIGURE 1. Osmotic accommodation to sea water of various salinities. Hydromedusae and ctenophores transferred from normal sea water to hyposaline sea water sink and remain on the bottom of the container even though swimming pulsations are not impaired. Conversely, animals transferred to hypersaline water float even though they, too, are able to pulsate normally. In time, animals in either hyposaline or hypersaline water adjust osmotically, regain their equilibrium buoyancy in the medium, and swim freely throughout the container.

was sufficient to compensate for their density. When placed in hypertonic sea water, all species initially floated. In addition to the change in relative buoyancy, animals placed in 60%, 75%, or 127% sea water usually responded behaviorally to the stress by contracting their bells and their tentacles; they usually ceased normal swimming pulsations for a few minutes. Eventually, in most cases, the medusae or ctenophores returned to their equilibrium buoyancies in the modified sea waters and carried out apparently normal activities.

The times required for individuals of each species to return to equilibrium buoyancy are given in Table I. Sea water diluted to 60% was lethal to most species; in such cases, individuals lay on the bottom, almost immediately ceased pulsating, and were unable to recover under any condition.

Most species accommodated within a few hours to 75% sea water, although initially the animals were relatively too dense to lift themselves off the bottom. Exceptions to this adaptability are listed below. *Aglantha* could not adjust, and indeed, usually succumbed in 75% sea water. *Phialidium* and *Aequorea* both failed to recover buoyancy and normal behavior in about one-half of the trials. *Gonionemus* usually did not behave normally in 75% sea water, but nevertheless was able to recover fully if placed back into normal sea water after 24 hours.

Sea water diluted to 90% caused a visible buoyancy problem for most species at first. Medusae were usually not able to lift off the bottom initially, but most were able to swim throughout the beaker within a few minutes. Complete return to equilibrium buoyancy in 90% sea water usually required a few hours—longer than resumption of swimming ability.

Medusae and ctenophores all floated after being introduced to hypertonic sea water of 127%, but all species (including those that are positively buoyant at equilibrium) began to sink within a few hours. It should be noted that most *Aglantha* and *Sarsia* and some *Bougainvillia* and *Phialidium* medusae never fully recovered equilibrium buoyancy and normal behavior in this high salinity water.

Even small changes in salinity caused initial sinking or floating of medusae. *Aequorea* and *Phialidium* required approximately 30–60 minutes to return to equilibrium buoyancy after being placed in water differing by only ±2‰ from normal sea water.

All medusae which had originally adjusted to hypo- or hypertonic sea water were able to readjust to normal sea water within 24 hours. The precise temporal details of this readjustment were not usually recorded.

Osmolality measurements of sea water samples and of mesogloea from 10 *Aequorea* medusae that had accommodated to the various sea water dilutions show that the osmolality of mesogloea closely corresponds over a wide range of salinities to the osmolality of the surrounding sea water (Fig. 2). In most cases these medusae were slightly hypo-osmotic to their surroundings.

All medusae placed in denser, but isotonic Percoll-sea water mixtures floated indefinitely at the surface. They were unable to swim very far below the water surface, but otherwise behaved normally for the entire 24 hour period of observation in this medium. When subsequently returned to normal sea water, all medusae immediately assumed their normal equilibrium buoyancies, confirming that no density adjustments had occurred in response to the denser Percoll-sea water medium.

DISCUSSION

These experiments demonstrate that many species of hydromedusae and ctenophores are able to adjust their buoyancies when they encounter changes in

TABLE I

Time in hours required for hydromedusae or ctenophores to adjust osmotically to water of various salinities

		Salinity—% of normal 30.5‰			
Species	Equilibrium buoyancy	60% (19‰ ± 1.0‰)	75% (23.0‰ ± 1.0‰)	90% (27.5‰ ± 1.0‰)	127% (38.0‰ ± 1.0‰)
Hydromedusae					
Aequorea victoria	negative	lethal; no recovery	20 or no recovery	1–5	5–10
Aglantha digitale	negative	lethal; no recovery	lethal; no recovery	no visible effect	1*
Bougainvillia principis	positive	not tested	4.5–6	1.5	1*
Gonionemus vertens	negative	buoyancy not recovered	24	1 or no visible effect	2–5
Phialidium gregarium	negative	lethal; no recovery	3 or no recovery	0.5–1.5	1–3
Polyorchis penicillatus	negative	6.5	7–11	2–5	3–6
Proboscidactyla flavicirrata	neutral	24	2–5	1–1.5	1–5
Sarsia tubulosa	positive	6 hours or no recovery	1–2.5	1–2	1–2*
Stomotoca atra	negative	lethal; no recovery	6–10	2.5–6	2.5–6
Ctenophora					
Bolinopsis infundibulum	negative	lethal; no recovery	3–20	3–8	3–8
Pleurobrachia bachei	negative	buoyancy not recovered	3–18	3–5	3–18

Animals were collected in sea water of 30.5‰ ± 1.0‰. Adjustment was measured as the resumption in new salinities of equilibrium buoyancy and normal behavior. * Indicates animals that were unable to recover their normal buoyancy, becoming heavier than normal, but otherwise behaved normally. The time listed for these animals reflects the number of hours required to change from positive to negative buoyancy.

salinity. Of the 9 hydromedusa and 2 ctenophore species tested, only *Aglantha* failed to adjust to changes of ±8‰ from 30‰ salinity. For the other species, individuals initially sink or float in the new medium depending on whether it is relatively hypo- or hypersaline; swimming behavior may be suppressed during this early period. Full buoyancy adjustment sometimes requires several hours, particularly if the salinity change is very great. Once accommodation has occurred, however, medusae and ctenophores display normal swimming and feeding activities.

Measurements of osmolality in *Aequorea* mesogloea imply that adjustment of

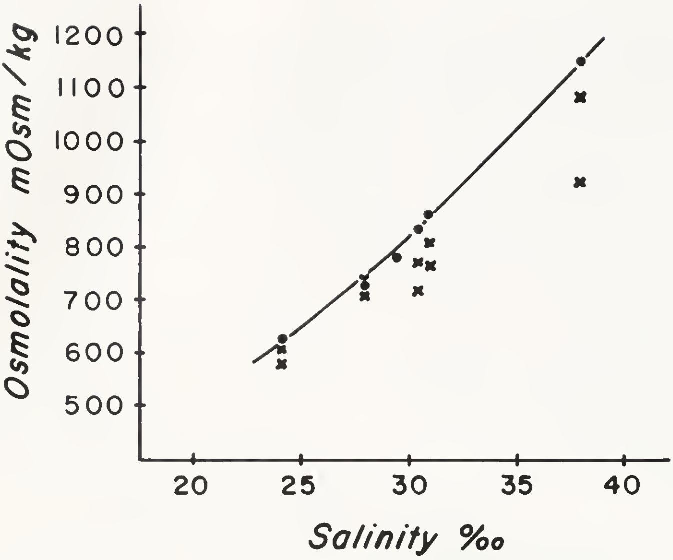

FIGURE 2. Osmolality of various dilutions of sea water (●) and of the mesogloea of 10 *Aequorea victoria* medusae (**X**) that had remained in the various sea water concentrations for 24 hours and regained their equilibrium buoyancies (curve is fit by eye, each point on the graph is established by two measurements of each sample).

buoyancy in this species is primarily the result of passive osmotic accomodation, *i.e.,* a mass movement of water between the inside and outside of a medusa based on osmolarity differences between the medusa and its surrounding medium. In terms of habitat, *Aequorea* is representative of those species that live in surface waters and are subject to large changes in salinity. Results with *Aequorea* probably can be extended to the other 10 species used in these experiments, but that are too small for conveniently measuring mesogloeal osmolality. (*Aglantha,* which did not readily adjust to salinity changes, lives in deep water—approximately 100 m by day. *Aglantha* is a vertical migrator, capable of moving up to the surface at night, but my results imply that *Aglantha* probably remains below any strong halocline.)

The Percoll experiments show that if sea water density is changed without altering salinity, jellyfish make no buoyancy adjustments in the medium. This is further evidence that changes in jellyfish buoyancy are not the result of active density regulation, but merely demonstrate a passive osmotic accommodation.

Changing the environment around a jellyfish in the laboratory certainly does not present the same situation as occurs when a jellyfish is moving through water with a salinity gradient, yet it allows conjecture about limitations to movement of medusae in the field. Accommodation as described above would occur if a jellyfish is suddenly confined to water of a different salinity, for instance if it is washed into a hypersaline lagoon or a diluted mixed estuary. Normally, however, in the nearshore habitats frequented by medusae and ctenophores, salinity changes are gradual or in small steps, with water of lower salinities layered over water of higher salinities.

Upon encountering stratification in the water column, the particular behavior of a jellyfish will depend on several variables: (1) steepness of the density gradient, (2) the rate of osmotic accommodation of the species, and (3) swimming behavior as the means by which a jellyfish propels itself into waters of different densities; it should be noted that most hydromedusae swim in bursts of several pulsations alternating with periods of inactivity.

An upward-swimming jellyfish, if the difference in densities at the discontinuity is so great that the jellyfish can no longer make upward progress, will sink back down into water of a density in which it can swim. If the jellyfish is positively phototactic, it will continue to swim up to the discontinuity; the jellyfish may eventually adjust to the overlying less-saline water because of frequent brief forays into it, until it finally accommodates and can continue to move upward. If the jellyfish has no strong photopositive response, it may move randomly away from the discontinuity before accommodation occurs, or it may remain just below the discontinuity. However, if the difference in salinities is small and the jellyfish can easily penetrate the discontinuity, it may accommodate to the less dense layer while swimming through it, regaining its equilibrium buoyancy. Passively sinking or rising jellyfish in the field should tend to accumulate at salinity discontinuities of as little as 1‰ until the animals begin to swim, so long as the density difference at the discontinuity is sufficient to oppose the natural buoyancy of the jellyfish.

Several laboratory studies have reported "behavioral" aggregations of various types of planktonic animals in the vicinity of artificially constructed salinity or temperature discontinuities in small columns. Such aggregations can be explained by physical as well as behavioral factors. Arai (1973, 1976) described an active behavioral aggregation at either temperature or salinity discontinuities by 2 species of hydromedusae (*Sarsia tubulosa* and *Phialidium gregarium*) and a ctenophore (*Pleurobrachia bachei*). Such aggregation was probably at least partially attributable to density differences between the medusae or ctenophores and their surrounding media. Because Arai totaled all observations during the second ½ hour of her observation period, osmotic accommodation which may have been taking place in jellyfish near the discontinuity during this period is not apparent in the published data. Lance (1962) reported the accumulation of copepods and crab zooea on both sides of a density discontinuity in the laboratory, with an increasing ability over time to penetrate the dilute upper layer. These observations are probably due to an increase of the relative density of organisms placed in dilute sea water and an initial inability of these organisms to propel themselves through the less dense medium, followed by gradual osmotic adjustment of density. Harder (1968) observed that a variety of planktonic animals in the laboratory accumulate at regular salinity discontinuities, but do not accumulate at salinity discontinuities where a density gradient is not also present (as when sucrose is added to the overlying less-saline layer). Again, the aggregation behavior at salinity discontinuities can probably be explained in terms of relative densities and swimming abilities. Hydromedusae and ctenophores are relatively large zooplankters and their movements across discontinuities are probably a major source of small-scale mixing.

There are no reports at present documenting cases in which jellyfish were able to pass through density discontinuities in the field. Cases in which medusae have been stopped by discontinuities have seemed more intrinsically interesting. Kramp (1959) states that diurnal movements of oceanic medusae are usually barred by a discontinuity. Hansen (1951) reports that a combined temperature and salinity discontinuity in Oslo Fjord separates the hydromedusa *Sarsia tubulosa,* which occurs only in the discontinuity layer, from the hydromedusa *Aglantha digitale* and the siphonophore *Lensia conoidea,* both of which appear to diurnally migrate within the water mass below the discontinuity. Moreira (1973, 1978) reports that salinity discontinuities off Brazil limit the upward vertical migration of some species of hydromedusae. Furthermore, temperature discontinuities off Brazil are also effective in limiting upward movement of some cold stenohaline species characteristic of deep subtropical waters, that are unable to tolerate the warm upper layers of shelf or coastal water.

In this paper, I have not studied the effects of temperature on buoyancy, but it is assumed that except when the water temperature above the thermocline is actually lethal, density differences caused by a thermocline should be fairly easily overcome *if* a jellyfish can actually swim across the barrier. Having a very large surface area and small overall mass, a medusa should rapidly equilibrate to the temperature of the surrounding water.

In summary, it has been demonstrated that medusae and ctenophores are able to osmotically accommodate most changes in salinity encountered in the sea. In the course of this adjustment, they regain their equilibrium buoyancy and normal swimming and feeding activities. This osmotic adjustment of buoyancy is apparently a separate process from the species-specific determination of equilibrium buoyancy via active exclusion of sulphate ion from the mesogloea.

ACKNOWLEDGMENTS

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EVIDENCE THAT ION REGULATION IN HYDROMEDUSAE AND CTENOPHORES DOES NOT FACILITATE VERTICAL MIGRATION

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ABSTRACT

Medusae and ctenophores, like many types of gelatinous zooplankton, actively exclude sulphate ion from their mesogloeal body fluid and thus gain lift (buoyancy). It is hypothesized that vertically migrating species might show day/night variations in the rate of sulphate elimination to regulate buoyancy dynamically and that such changes are a major factor in vertical migration. To test this hypothesis, a series of laboratory experiments were conducted using medusae and ctenophores. Concentrations of radioactive sulphate were measured in equilibrium (uptake) experiments and concentrations of sodium, magnesium, potassium, and calcium ions were measured using atomic absorption spectrophotometry. No evidence of day/night (light/dark) changes in ion concentrations were found for the hydromedusae *Aequorea victoria, Aglantha digitale, Gonionemus vertens, Mitrocoma cellularia, Phialidium gregarium, Polyorchis penicillatus, Sarsia tubulosa,* and *Stomotoca atra* or for the ctenophore *Pleurobrachia bachei.* It is concluded that changes in ionic regulation are not a major factor in diel vertical migration. It is thus hypothesized that for these animals vertical migration is accomplished solely by swimming.

INTRODUCTION

Jellyfish, like many planktonic organisms, are often restricted to relatively narrow depth ranges in the sea. These distributions may be modified diurnally for species that undergo vertical migrations, moving toward the surface at night and into deeper water during the day. One of the most impressive vertical migrations of a hydromedusa is that of *Solmissus albescens* in the Adriatic Sea (Benović, 1973). This jellyfish, which is usually less than 3 cm in diameter, moves up and down more than 300 m each way daily. The hydromedusa *Aglantha digitale* performs the most extensive vertical migration among the jellyfish in the Puget Sound—Strait of Georgia region where the present work was done, moving up and down as much as 100 m each way daily (Arai and Fulton, 1973; Mills, 1982).

In order to maintain themselves consistently at certain depths, planktonic animals must either attain neutral buoyancy, somehow balancing the weight of their proteinaceous tissues, or they must establish swimming routines to overcome buoyancy differentials. Most hydromedusae in the Puget Sound—Strait of Georgia region are not neutrally buoyant. At rest in surface waters, most species sink approximately 20–120 cm per minute (Mills, 1981 and unpub. obs.). Only a relatively small number of the local species of hydromedusae are either neutrally or positively buoyant. Of

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the negatively buoyant species, most have intermittent swimming patterns of short duration that serve both to maintain a given depth in the water column and simultaneously to facilitate feeding (Mills, 1981).

The mesogloea, or jelly, of a hydromedusa is a highly hydrated, acellular fibrous material. Several studies have shown that the concentrations of major ions in cnidarian mesogloea are generally similar to sea water, with one major exception—the concentration of sulphate ion within the mesogloea is much lower than in the surrounding sea water (Macallum, 1903; Koizumi and Hosoi, 1936; Robertson, 1949). Chloride concentration in the mesogloea is somewhat higher than in sea water, apparently as a result of the isosmotic replacement of sulphate ions by chloride ions (Robertson, 1949). The work of Robertson (1949) and Mackay (1969) suggests that excretion of sulphate ion (along with associated cations) in jellyfish is accomplished by an active transport system across the epithelium.

Elimination of sulphate from the body fluids of gelatinous planktonic organisms provides buoyancy. Denton and Shaw (1962) and Bidigare and Biggs (1980) have calculated that partial elimination of sulphate ions and isosmotic replacement with chloride provides enough lift to balance some or all of the relatively heavy proteinaceous tissue. For example, Bidigare and Biggs (1980) found that in the ctenophore *Beroe cucumis,* elimination of 55% of the sulphate relative to sea water provides 1.2 mg of lift per ml of mesogloeal volume, offsetting all of its protein mass and rendering this weak swimmer neutrally buoyant. The more robust scyphomedusa *Pelagia noctiluca* eliminates 38% of the sulphate relative to sea water, gaining 0.8 mg of lift per ml of mesogloeal volume and offsetting only 66% of its protein mass; unlike *Beroe, Pelagia* is a strong swimmer and has no difficulty in overcoming its residual negative buoyancy by active swimming. Thus sulphate elimination may either completely or only partially offset the heavy components in a jellyfish. The final buoyancy is apparently the result of morphology (amount and distribution of heavy and buoyant tissues) and a trade-off between energy used in swimming and that used in sulphate elimination.

Given that a low sulphate concentration in the mesogloea is maintained by an active transport system, the question arises as to whether vertically migrating medusae can diurnally vary the rate of sulphate elimination, thereby facilitating vertical migration by actively regulating buoyancy. It has not yet been established whether vertical migration of medusae is accomplished solely by swimming, or solely by regulation of buoyancy, or by a combination of both mechanisms (Mackie, 1974). Extensive observations of hydromedusae in a 1500 l aquarium (Mills, 1981, 1983) however, have not revealed any visible changes in buoyancy between day and night. Thus, if buoyancy is regulated diurnally, the magnitude of change may be insufficient by itself to drive migration; on the other hand, even a small change in buoyancy would facilitate swimming.

The purpose of the present paper is to test empirically the hypothesis that sulphate concentration in jellyfish varies on a day/night basis in such a way that vertical migration might be facilitated. Accordingly, we have determined the concentrations of sulphate and of several other ions in whole medusae and have compared values for animals held in the light and dark. Measuring ions other than sulphate served as a secondary method for investigating possible day/night ion-controlled buoyancy changes, since concentration changes of any ion are expected to be accompanied by changes in one or more counterions in order for the jellyfish to remain in osmotic equilibrium with sea water. In previous experiments (Mills, 1983), it was established that rhythms of vertical migration in medusae are responses to light and dark rather than to intrinsic clocks. That is, medusae held in continuous light (or dark) for long periods continue to behave appropriately for that light condition. Subsequent changes in lighting elicit abrupt changes in migratory behavior which are appropriate to the new light condition.

MATERIALS AND METHODS

Animals

In all experiments it was considered of paramount importance that the medusae be in excellent condition and that they be handled as little as possible during the experiments. Most of the animals were collected individually in beakers from the floats in front of the Friday Harbor Laboratories. *Gonionemus* medusae were collected by the same procedure in Mitchell Bay, San Juan Island, Washington, and *Polyorchis* medusae were collected from Shoal Bay, Lopez Island, Washington, and from Bamfield Inlet, British Columbia. Animals were always collected during the day and had always experienced at least 8 hours of light prior to being placed in the dark. Medusae were gently transferred to larger containers of sea water and were maintained under a regulated light regime in an 11°C walk-in coldroom at the Friday Harbor Laboratories. Experiments were begun within a few hours of collection.

To test the ion regulation/vertical migration hypothesis, species representing various diel behavior patterns (Mills, 1982) were selected for this study. *Aglantha digitale* is a long-range vertical migrator that moves as much as 100 m each way daily. *Gonionemus vertens* lives in bays only a few meters deep, but nevertheless has a pronounced migration, spending the daytime mostly attached to vegetation on or near the bottom and spending the night swimming near the surface. *Polyorchis penicillatus* also lives in shallow bays, but does not exhibit such a pronounced vertical migration. *Polyorchis* medusae swim up and down all the time, but they seem to spend more time near the surface at night. *Stomotoca atra, Phialidium gregarium,* and *Aequorea victoria* all tend to remain in the uppermost 25 meters. Here *Stomotoca* appears to make a short-range vertical migration; *Phialidium* apparently makes a short-range reverse vertical migration (moving down at night); and *Aequorea* shows no evidence of a day/night pattern. *Pleurobrachia bachei* apparently migrates upward within the upper 50 m at night. Diel behaviors of *Mitrocoma cellularia* and *Sarsia tubulosa* are not known.

Sulphate protocol

Sulphate concentrations of whole medusae were investigated using radioactive sulphate ($Na_2{}^{35}SO_4$) in order to establish whether the concentrations of sulphate attained under light *versus* dark conditions were different. The following six species of hydromedusae were incubated in natural sea water plus 40–200 μCi/l of $Na_2{}^{35}SO_4$ in constant light or in constant dark as described in Table I: *Gonionemus vertens, Mitrocoma cellularia, Phialidium gregarium, Sarsia tubulosa, Aglantha digitale,* and *Aequorea victoria.*

Shortly after collection, medusae were transferred to small paper cups or glass beakers containing natural sea water plus labeled sulphate. For 4 of the 6 species, some medusae were left under a fluorescent room light and others were placed in a dark drawer in the same room. All *Aglantha* and *Aequorea* medusae were left in the light because only small numbers of these animals were available.

Medusae in the light were sampled intermittently to establish equilibrium values for each species (Fig. 1). Sulphate concentrations in *Sarsia* and *Aequorea* did not equilibrate during the experimental period (45 and 76 hours, respectively) and sampling was terminated when the remaining individuals began to look unhealthy. Medusae

TABLE I

Conditions of radioactive sulphate saturation experiments for 6 species of hydromedusae held in constant light or constant dark

Species name	Length of experiment (hours)	Time to equilibrate (hours)	$\mu Ci^{35}SO_4$ per liter SW	# medusae per volume SW	Wet weight of single medusae (g)
Gonionemus vertens	66	≤20	66	2/75 ml	0.220–1.186
Mitrocoma cellularia	109	≤85	40	2/500 ml	1.00–2.74
Phialidium gregarium	45	≤8	66	2/50 ml	0.086–0.463
Sarsia tubulosa	45	≥45	66	2/50 ml	0.054–0.360
Aglantha digitale	54	≤4	200	2/50 ml	0.017–0.155
Aequorea victoria	76	≥76	200	1/75 ml	0.874–3.300

Time to equilibrate was determined from plots of sulphate concentrations in Figure 1.

which had been kept in the dark were analyzed only at the final time point for medusae of the same species held in the light, for the purpose of comparing light *versus* dark sulphate concentration at that time. Each medusa was removed from the labeled sea water, carried through 2 rinses of fresh sea water, blotted "dry" on a paper towel for 5–15 seconds depending on the species, and then was homogenized through a 20-gauge syringe needle into a preweighed vial. Each vial was immediately capped and reweighed to determine the wet weight of the medusa. In order to determine the amount of radioactive sulphate remaining in each container, two 0.5 ml samples of radioactive water were taken to accompany each medusa, and were placed in separate vials. Three ml of Aquasol scintillation fluid (New England Nuclear, Inc.) were added to all vials. All samples were counted for 5 minutes on a Beckman LS8000 liquid scintillation counter using a broad C^{14} window. Radioactive counts ranged between 2000 cpm (in *Gonionemus* and *Mitrocoma*) and 40,000 cpm (in *Phialidium*). Values were not adjusted for quenching. Radioactivities of the medusae relative to the sea water, after adjustment for the blotted wet weights of the medusae, were then calculated as cpm per ml of jellyfish divided by cpm per ml of sea water. Student's *t*-test was used to compare light and dark mean values at the final time point for all species except *Aglantha* and *Aequorea* (for which no dark values were determined due to a shortage of animals).

Sodium, magnesium, potassium, and calcium protocol

Concentrations of sodium, magnesium, potassium, and calcium ions in whole medusae maintained in either light or dark were measured using a Varian AA-475 series atomic absorption spectrophotometer. Ten individuals each of 6 species of medusae (*Aequorea victoria, Gonionemus vertens, Mitrocoma cellularia, Stomotoca atra, Polyorchis penicillatus,* and *Phialidium gregarium*) and of one ctenophore (*Pleurobrachia bachei*) were carefully hand collected and transferred into 500–1000 ml beakers (5 medusae per beaker) in an 11°C walk-in coldroom. Medusae were collected in late morning or early afternoon and then held in fluorescent light until mid-afternoon when all had experienced 10–14 hours of "daylight." At this time, for each species, one-half of the animals were placed in a dark drawer and one-half remained in the light. After 2 hours, both sets of medusae were processed as follows. Each medusa was picked up gently with forceps and blotted "dry" on a paper towel for 5 to 15 seconds, depending on the species. The whole medusa (or in the cases of

Aequorea, Mitrocoma, and *Polyorchis,* where the animals were large, ¼ of a whole medusa was used) was then placed in a preweighed glass scintillation vial and the weight of the medusa was recorded. Four 1.0 ml sea water samples were also taken for comparison. One ml of analytical grade nitric acid was then added to each vial in order to dissolve the medusa, and the vials were capped with polyethylene-lined lids. Some of the samples required gentle heating on a hotplate for about 20 minutes to dissolve fully the animal tissue.

For A.A. spectrophotometric measurements, the dissolved medusae were diluted to 100 ml (and secondarily diluted up to 500 ml where necessary) in volumetric flasks using ultrapure water from a Sybron/Barnstead NANOpure filter with measured conductivity less than 7.0 megohms/cm. Standard solutions were made up using ultrapure water as follows: 20, 100, 200 ppm sodium; 2, 10, 20 ppm magnesium; 0.8, 4, 8 ppm calcium; and 0.8, 4, 8 ppm potassium. Sodium and potassium were measured from emission, and magnesium and calcium were measured from absorption. Concentrations of these ions were converted from ppm (μg/ml of sample) to μg/g wet weight of jellyfish. The ion concentrations in the jellyfish were subsequently compared to ion concentrations in Friday Harbor sea water (ppm in the animal × 100 divided by ppm in sea water) in Figures 3–6.

RESULTS

Sulphate

Sulphate concentrations are shown for 6 species of hydromedusae (Fig. 1). The concentration in *Gonionemus vertens* appears to have equilibrated within about 20 hours, with a tissue sulphate concentration equivalent to 8% of the concentration in the external sea water. *Mitrocoma cellularia* had apparently equilibrated by 85 hours, containing an average of 24% of the external sulphate although no intermediate points were taken for this species. *Phialidium gregarium* appears to have equilibrated in less than 10 hours, containing an average of 77% of the external sulphate. *Sarsia tubulosa* also had not obviously reached equilibration by 45 hours when sampling of this species was terminated because the animals were beginning to look unhealthy; at that time they contained an average of 27% of the external sulphate. *Aglantha digitale* had apparently equilibrated by the time the first samples were taken at 4 hours, containing an average of 28% of the external sulphate. *Aequorea victoria* had not obviously reached equilibration by 76 hours, when it contained 87% of the external sulphate.

Sulphate levels in both light and dark were obtained for all of the above species of hydromedusae except *Aglantha* and *Aequorea.* Mean light and dark values for each species are plotted in Figure 2. For *Gonionemus, Mitrocoma, Phialidium,* and *Sarsia,* two-tailed *t*-tests show no significant difference in sulphate uptake levels in continuous light *versus* levels in continuous dark.

Sodium, magnesium, potassium, and calcium

Mean ion concentrations in whole animals (6 species of hydromedusae and 1 ctenophore) after 2 hours in the light or after 2 hours in the dark are shown in Table II and Figures 3–6. Although the 10 individuals of each species were of various sizes, the standard deviations were very low. Student's *t*-test failed to reveal significant differences in any case between ion concentrations in the light and after 2 hours of darkness.

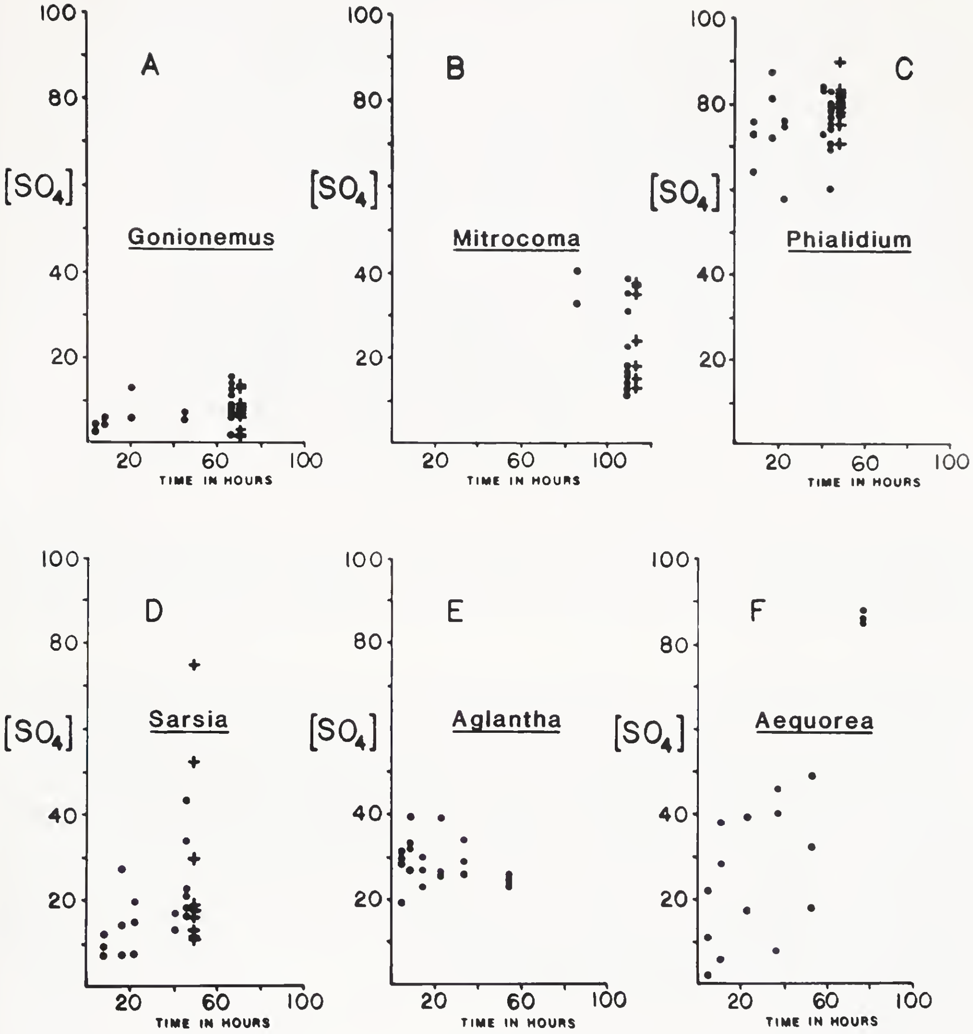

FIGURE 1. Uptake of sulphate ($Na_2{}^{35}SO_4$) in 6 species of hydromedusae in constant light (●) or dark (+). Sulphate levels are expressed as specific activity of the medusa (cpm/ml) × 100 divided by specific activity of the sea water (cpm/ml). (A) *Gonionemus vertens,* (B) *Mitrocoma cellularia,* (C) *Phialidium gregarium,* (D) *Sarsia tubulosa,* (E) *Aglantha digitale,* (F) *Aequorea victoria.*

DISCUSSION

Within the sensitivities of the methods used, there is no evidence for a general phenomenon of light/dark (day/night) variation in the concentrations of sulphate, sodium, magnesium, potassium, or calcium ions in the hydromedusae and ctenophores examined. In nature, it is dark for only 4 to 5 hours during mid-summer when these studies were conducted. Medusae migrate upward shortly after dusk and downward at dawn, so any useful change in buoyancy should be indicated during the first hour of darkness or light. Although the radioactive sulphate experiments necessarily occurred over an unnaturally long period of uninterrupted light or dark, agreement of these

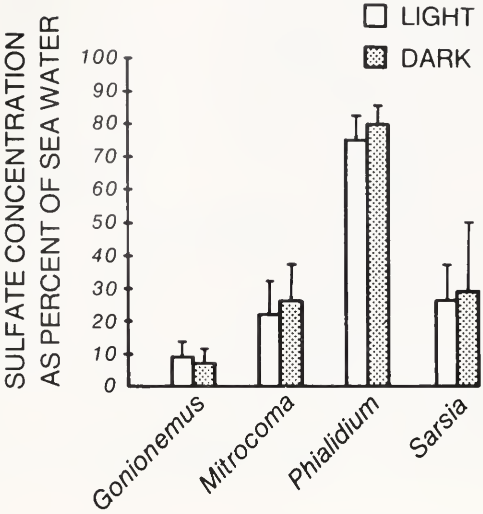

FIGURE 2. Sulphate concentrations of 4 species of hydromedusae in the light and dark relative to sulphate concentrations in sea water. Each bar represents the mean of 6–12 samples (see Figure 1 for precise sample sizes). One standard deviation is indicated above each bar.

results with the more realistic light/dark time periods used for medusae in which other ion concentrations were determined lends strength to the former studies. Since the five measured ions do not vary between day and night, it is implied that probably no ions vary substantially between day and night, because one would expect that change in one ion should be balanced by an opposing change in one or more other ions. Furthermore, it is unlikely that, if ionic movements are involved in producing day/night differences, other cations are involved, because with the exception of ammonium, all major sea water cations were checked. It is conceivable that an anion other than sulfate might be actively transported, creating density differences over the 24 hour cycle, but no examples of buoyancy regulation by anions other than sulphate are known, so this possibility is rather remote.

TABLE II

Ion concentrations in whole medusae after approximately 15 hours of light (L) or after 2 hours of darkness following approximately 13 hours of light (D)

Species	Wet weight (g)	Sodium (μg/g)		Magnesium (μg/g)		Calcium (μg/g)		Potassium (μg/g)	
		L	D	L	D	L	D	L	D
Aequorea victoria	3.756–6.598	9414	9308	1054	1051	485.2	493.8	429.2	382.4
Gonionemus vertens	0.659–1.187	8170	7797	666.5	673.4	332.2	333.6	1418	1491
Mitrocoma cellularia	2.906–4.548	9469	9502	988.2	965.6	436.2	425.4	652.2	649.4
Phialidium gregarium	0.163–0.517	9665	9747	1017	1041	442.6	447.8	426.4	427.6
Polyorchis penicillatus	1.411–4.552	8777	8633	943.8	943.2	515.0	527.8	1313	1213
Stomotoca atra	0.249–0.573	9280	8970	800.5	825.4	444.5	444.2	653.2	655.0
Pleurobrachia bachei	0.521–1.083	9284	9221	969.4	976.6	464.8	417.6	439.8	426.8
Sea water (1 ml)	1.028–1.051	9326		1216		471.0		355.0	

Values given are the means of 5 samples; standard deviations are shown in Figures 3–6.

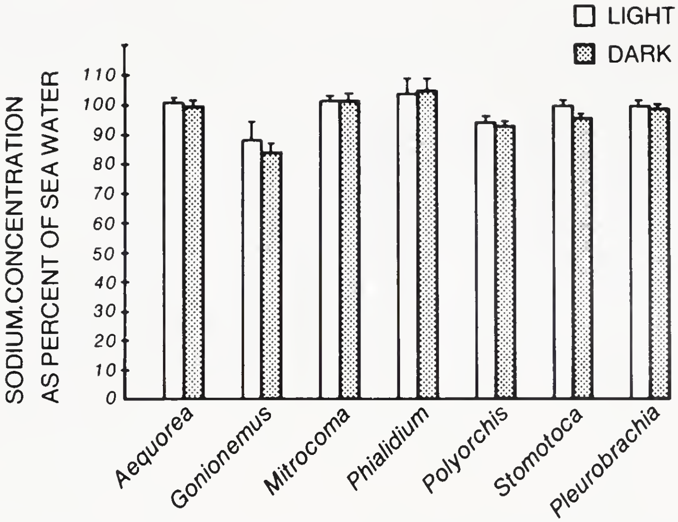

FIGURE 3. Sodium concentrations of 6 species of hydromedusae and 1 ctenophore in the light and dark relative to sodium concentration in sea water. Each bar represents the mean of 5 samples. One standard deviation is indicated above each bar.

The species of jellyfish used in this study represent a diverse set of diel behavior patterns (see *Animals* above). Since day/night ion concentrations in medusae do not vary in accordance with diel activity patterns, it is postulated that vertical migration is apparently accomplished by swimming and not by diel ionic regulation of buoyancy. Alternatively, it is conceivable that diel changes in feeding could allow the use of gut contents to function as a buoyancy factor for vertical migration. *Phialidium, Mitro-*

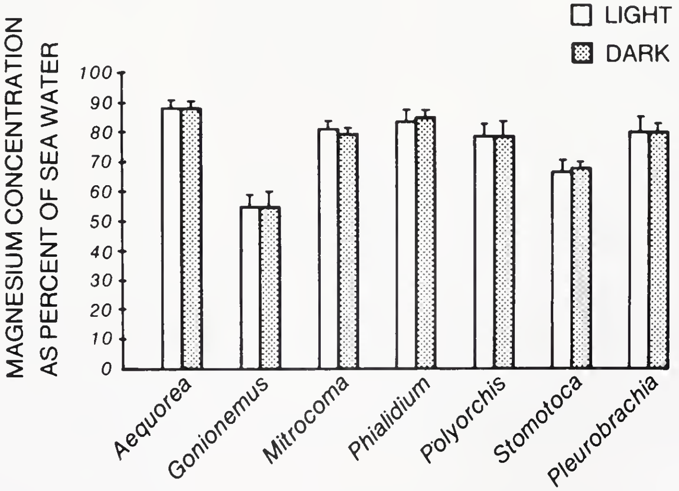

FIGURE 4. Magnesium concentrations of 6 species of hydromedusae and 1 ctenophore in the light and dark relative to magnesium concentration in sea water. Each bar represents the mean of 5 samples. One standard deviation is indicated above each bar.

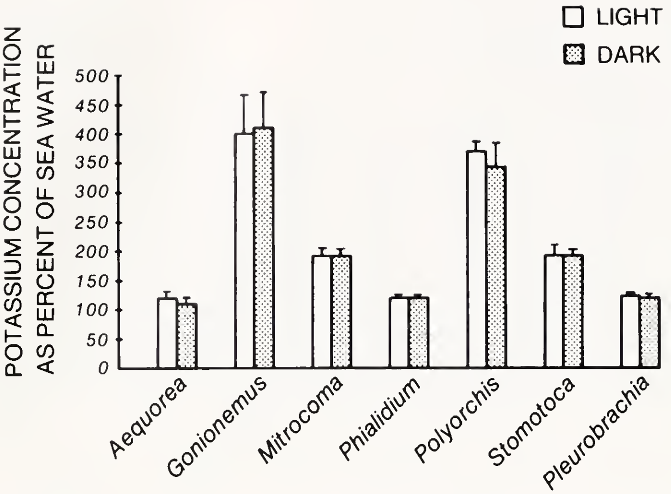

FIGURE 5. Potassium concentrations of 6 species of hydromedusae and 1 ctenophore in the light and dark relative to potassium concentration in sea water. Each bar represents the mean of 5 samples. One standard deviation is indicated above each bar.

coma, and *Aequorea* medusae in a 1500 l aquarium have been observed to gradually change from negative to neutral buoyancy over a period of several days without food (unpub. obs., C.E.M.). This hypothesis has not been systematically investigated.

Although no diel changes in ion levels have been discovered, it seems likely that

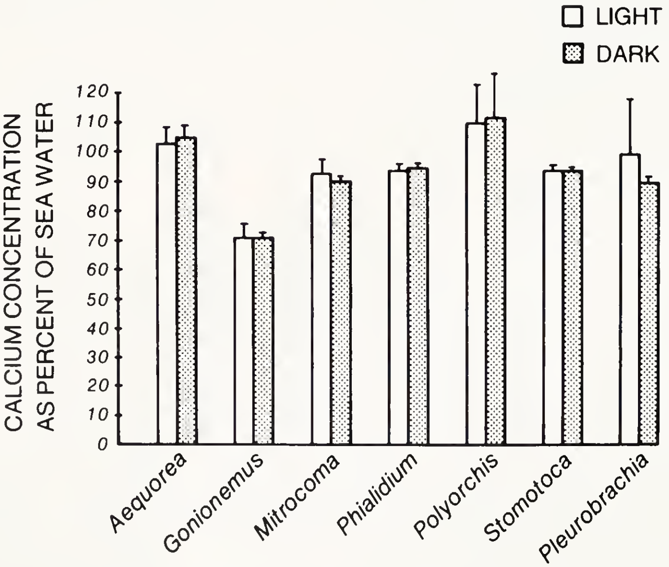

FIGURE 6. Calcium concentrations of 6 species of hydromedusae and 1 ctenophore in the light and dark relative to calcium concentration in sea water. Each bar represents the mean of 5 samples. One standard deviation is indicated above each bar.

continuous exclusion of sulphate is used as a means of obtaining lift perhaps universally by planktonic Cnidaria and ctenophores. Figure 7 summarizes the findings of 8 different papers published over the past 80 years that have reported sulphate concentrations in either whole animals (this study only) or in fluid extracts of the mesogloea. Different techniques for sulphate analysis were used in each study, but in most cases the results are very similar. Mackay (1969) found that no significant sulphate binding occurred in mesogloea and that all of the mesogloeal sulphate ion was exchangeable, so measurements of total sulphate and of labeled sulphate uptake should be comparable. Various authors reported exclusion of 2–92% of the available sulphate in sea water; most of the animals in nature were either neutrally buoyant or slightly negatively buoyant. For most species of pelagic coelenterates and ctenophores, exclusion of sulphate is probably the most important source of lift.

Ammonium ions, when concentrated and retained within tissues, are important in the floatation systems of some marine organisms including dinoflagellates (Singarajah, 1979), tunicate eggs (Lambert and Lambert, 1978), and certain mid-water squid (Denton *et al.*, 1958). Ammonium is present only in very low levels in planktonic Cnidaria and ctenophores (Singarajah, 1979; Bidigare and Biggs, 1980) and apparently makes little contribution to buoyancy in these animals. Some species of hydromedusae (*e.g., Aglantha digitale* and *Plotocnide borealis*) have a relatively large oil droplet that undoubtedly contributes to the buoyancy of these animals, but because these species are not abundant, lipid contributions to buoyancy have not been analyzed in this study. A general review of the various buoyancy mechanisms employed by marine organisms is given by Denton (1963).

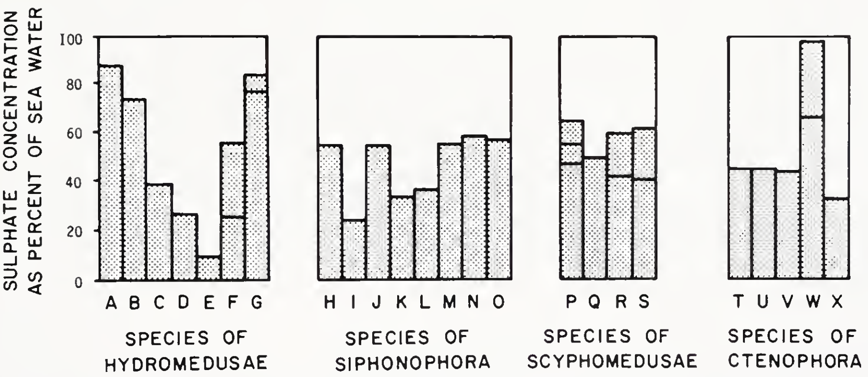

FIGURE 7. Sulphate concentrations of hydromedusae, siphonophores, scyphomedusae, and ctenophores expressed as percent of the sulphate concentration in natural sea water showing concentration in the jellyfishes consistently less than in sea water. Species names and references for sulphate measurements are as follows:

A, *Aequorea coerulescens*[2]; B, *Aequorea victoria* (as *A. aequorea*)[5]; C, *Aequorea aequorea* (as *A. forskalea*)[4]; D, *Aglantha digitale*[8]; E, *Gonionemus vertens*[8]; F, *Mitrocoma cellularia* (also as *Halistaura cellularia*)[5,8]; G, *Phialidium gregarium*[5,8]; H, *Agalma okeni*[7]; I, *Diphyes dispar*[7]; J, *Forskalia edwardsi*[7]; K, *Hippopodius hippopus*[7]; L, *Rosacea cymbiformis*[7]; M, *Stephanophyes superba*[7]; N, *Vogtia glabra*[7]; O, *Vogtia spinosa*[7]; P, *Aurelia aurita* (also as *A. flavidula*)[1,3,7]; Q, *Chrysaora melanaster* (as *Dactylometra pacifica*)[2]; R, *Cyanea capillata* (also as *C. arctica*)[1,2]; S, *Pelagia noctiluca*[4,7]; T, *Beroe cucumis*[7]; U, *Beroe forskalia*[4]; V, *Beroe ovata*[4]; W, *Cestum veneris*[4,7]; X, *Pleurobrachia pileus*[6].

[1] Macallum (1903).
[2] Koizumi and Hosoi (1936).
[3] Robertson (1949).
[4] Denton and Shaw (1962).
[5] Mackay (1969).
[6] Singarajah (1979).
[7] Bidigare and Biggs (1980).
[8] Mills (this report).

The rates of sulphate uptake varied widely in the 6 species examined here (Fig. 1), ranging from less than 4 hours for achieving equilibrium in *Aglantha* to over 75 hours for achieving equilibrium in *Aequorea* and *Mitrocoma.* For species measured in common, our equilibrium times and values are similar to those of Mackay (1969). He interpreted the differences in uptake rate as a reflection of interspecific differences in permeability to sulphate, using specimens of similar sizes so that direct interspecific comparisons could be made. In contrast, we used individuals of different sizes within each species (wet weights ranging nearly 3× in *Mitrocoma* up to 9× in *Aglantha*), yet the intraspecific equilibrium times and values still are fairly constant. This further supports the concept of species-specific sulphate permeability.

Among the other ions measured, potassium exhibits the greatest variation in concentration between species of jellyfish (Figs. 3–6). Because we used whole jellyfish in our study, the high potassium concentrations probably reflect high intracellular potassium, and interspecific differences are presumably indicative of amounts of cellular tissue such as tentacles and gonad relative to the amount of jelly.

Species-specific sulphate concentrations appear to relate loosely to morphology and activity levels, rather than to vertical migration, as originally suspected. For example, *Gonionemus,* which has up to 80 thick tentacles and 4 large gonads (and the greatest potassium concentration), also has the greatest capability for sulphate exclusion. *Gonionemus* is still negatively buoyant, but is a strong swimmer and so can overcome the buoyancy differential. On the other hand, *Sarsia* has only 4 tentacles and a single gonad and is somewhat less efficient at excluding sulphate, yet it achieves positive buoyancy, perhaps because it has less cellular tissue. It appears that hydromedusae have evolved species-specific swimming and feeding patterns based largely on this balance of intrinsic buoyancy and morphology (Mills, 1981).

Buoyancy maintainance that is derived from an active transport system such as the exclusion of sulphate ions or the accumulation of ammonium ions, requires an expenditure of metabolic energy. In the case of tunicate eggs which float due to an accumulation of ammonium ions in follicle cells, Lambert and Lambert (1978) used a variety of metabolic inhibitors to demonstrate unequivocally that the energy for this process comes from glycolysis rather than oxidative phosphorylation. The metabolic basis for ion transport in Cnidaria and ctenophores remains to be determined, as well as the relative energy expenditure for ion pumping *versus* swimming in these organisms.

ACKNOWLEDGMENTS

This work was supported by a University of Victoria Graduate Fellowship, Canadian NSERC Grant #G0226 to Dr. G. O. Mackie and NSF Grant #OCE8214058 (C.E.M.), and by NIH National Research Service Award #GM07270 (R.G.V.). Special thanks to Dr. T. Fyles for assistance with the A.A. spectrophotometer.

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SYNCHRONIZED RHYTHMIC CONTRACTIONS AMONG FIVE GONADAL LOBES IN THE SHEDDING SEA URCHINS: COORDINATIVE FUNCTION OF THE ABORAL NERVE RING

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ABSTRACT

The gonadal lobe of the spontaneously shedding sea urchin, *Temnopleurus toreumaticus,* contracts rhythmically. The gonadal movement following electrical or KCl stimulation is a summed response of the rhythmic and arrhythmic contractions. The lobes pretreated with $MgCl_2$ show only the latter type. Contraction induced by acetylcholine is the latter type. The aboral nerve ring (ANR) connects proximal ends of five gonoducts. In a preparation with intact ANR, the rhythmic contraction occurs synchronously in all five gonadal lobes. When the ANR is separated into two sectors, lobes belonging to the same sector contract synchronously, but those belonging to the different sectors do not.

These results demonstrate rhythm generators (pacemakers) in the ANR that are responsible for individual and synchronous contractions among the gonadal lobes.

INTRODUCTION

Eggs and sperm of sea urchins are used widely in various fields of modern biology. These gametes are routinely obtained by injecting isotonic KCl solution (Palmer, 1937; Harvey, 1939) or acetylcholine (ACh) dissolved in sea water (Iwata and Fukase, 1964a) into the test cavity, or by stimulating the animal electrically (Iwata, 1950, 1976; Harvey, 1952). Iwata and Fukase (1964b) speculated that both KCl and electrical stimulation directly affect the gonadal muscle (Palmer, 1937; Kawaguti, 1965), while ACh stimulates its cholinergic receptors.

Iwata (1976) reported that an increase in the internal pressure of gonads, possibly due to muscular contraction, results in spontaneously or artificially induced shedding.

Therefore, we investigated the shedding process by simultaneously recording gonadal contractions in five gonadal lobes after surgery on putative neural pathways. The results clearly show that the rhythmicity in the contraction pattern is synchronized among lobes and that the synchronization is achieved by coordination of neural activities in the aboral nerve ring (ANR).

MATERIAL AND METHODS

Sea urchins [*Temnopleurus toreumaticus* (Leske)], 3 to 5 cm in test diameter, were collected in the Seto Inland Sea near the Ushimado Marine Laboratory of Okayama University. Experiments were carried out mostly during the breeding season from July to August. No sexual differences were found in the response pattern.

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Abbreviations: ACh, acetylcholine; ANR, aboral nerve ring.

The animals were cut along the equator of the test and the gonad bearing aboral halves (vol. 3 to 7 ml) was used. All spines were scraped off with rough whetstone to avoid any mechanical disturbance. Digestive canals were cut with a razor blade.

A 2.5 cm diameter hole was made in the center of a horizontal shelf found on the upper portion of an acrylic experimental vessel (10 × 10 × 10 cm). The specimen was placed aboral side down on the hole and the cup-shaped test was filled to the upper edge of the gonads with sea water.

A straw placed vertically on the central surface of the gonadal lobe was connected to a high-gain force displacement transducer (Nihon Kohden, SB-1T-H) and the ensuing potential changes were amplified (Nihon Kohden, RP-3). Three sets of apparatus were used to simultaneously record contractions in three out of the five gonadal lobes on a multichannel pen-recorder (Nihon Kohden, RJG-3024).

Internal pressure changes were measured by placing a sea water filled glass tube, 2 mm in internal diameter, on one of the five genital pores. The space between the genital pore and the glass tube was sealed with rubber tubing. The glass tube was connected to a high-gain pressure transducer (Nihon Kohden, LPU-0.1), coupled to a pre-amplifier (Nihon Kohden, RP-3).

Repetitive electrical stimuli (10 ms, 50 Hz, 20 to 30 V) were applied for 5 to 20 seconds through a pair of silver-silver chloride electrodes (0.6 mm in tip diameter), one being placed inside the test and the other in the sea water outside the preparation. Thus the stimulation current flowed mainly through the aboral region of the test. To stimulate a gonadal lobe directly, both electrodes were placed on the surface of the lobe.

Stimulating reagents consisted of 0.5 M KCl and 10^{-4} M ACh dissolved in sea water. A few drops of these reagents were added to the preparation. To block neuromuscular transmission, the test cavity was filled with isotonic $MgCl_2$ for 15 minutes and the solution was replaced by sea water immediately before the experiments.

RESULTS

Rhythmic contractions of the gonad

Figure 1A demonstrates the twitch-like contractions during spontaneous gamete discharge. In the absence of gamete shedding, the contraction heights were much smaller. Identical temporal coincidence was observed between the contraction pattern and the internal pressure changes of the gonad (Fig. 1B).

Electrical stimulation of the aboral region induced tetanic contractions in gonads, resulting in a vigorous gamete discharge (Fig. 2A). Those responses were always accompanied by small rhythmic contractions. Usually the gonads ceased to contract a few minutes after the stimulation. Gonads responded to ACh by an arrhythmic contraction (Fig. 2C), which became rhythmic upon electrical stimulation of the aboral region (Fig. 2D). The addition of KCl caused the gametes to shed copiously and the gonad showed tetanic contractions accompanied with rhythmic contractions (Fig. 2E).

Rhythmic and arrhythmic contractions

Arrhythmic and spatially restricted contractions were evoked when electrical stimulation was given directly to the gonadal surface. The rising and falling phases of those arrhythmic responses were very slow.

The possibility of a chemical secretion being involved in the rhythmic contractions was tested using preparations pretreated with $MgCl_2$ in order to block synaptic trans-

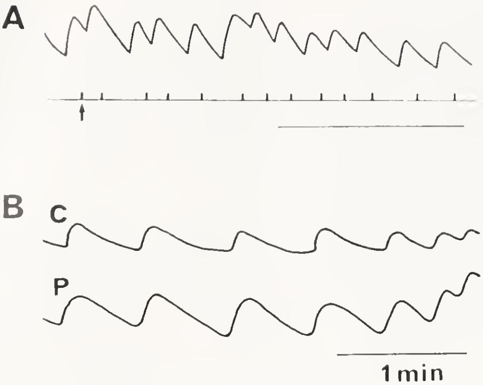

FIGURE 1. Temporal relationships among three spawning-related events. A: Rhythmic contractions of the gonadal lobe (upward deflections in the upper trace) and heavy shedding observed with the naked eye (arrow). B: Rhythmic contractions (C) and changes in the internal pressure of the lobe (P).

missions (Fig. 3). Gamete discharges were observed in response to suprathresholdal electrical stimulation. The response to the electrical stimulation of the surface of the lobe resembled that observed in the intact preparation (Fig. 3A). On the other hand,

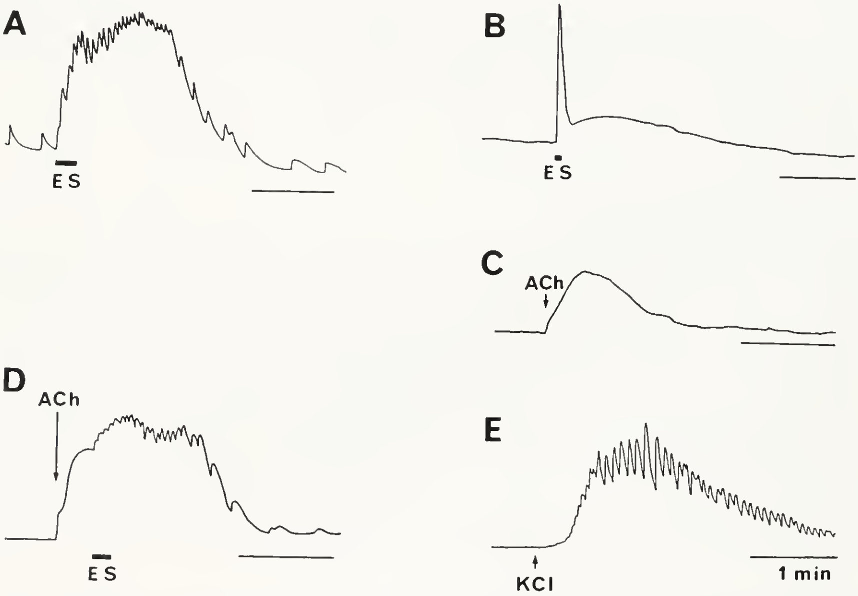

FIGURE 2. Five types of contraction pattern. A: Spontaneously and rhythmically contracting lobe responds by an incomplete tetanus-like contraction to electrical stimulation (ES) given through the aboral region of the test. Note that small rhythmic contractions persist with higher frequency. B: Lobe deprived of its own gonoduct responds to electrical stimulation (ES) to aboral region with bi-phasic contraction; initial fast twitch-like contraction is followed by an arrhythmic slow one. C: Arrhythmic contraction in response to 10^{-4} *M* ACh. D: The arrhythmic contraction induced by ACh becomes rhythmic when electrically stimulated (ES). E: Response of a quiesent lobe of the addition of a few drops of 0.5 *M* KCl into the test cavity. Note that small rhythmic contractions are superposed on the arrhythmic contraction.

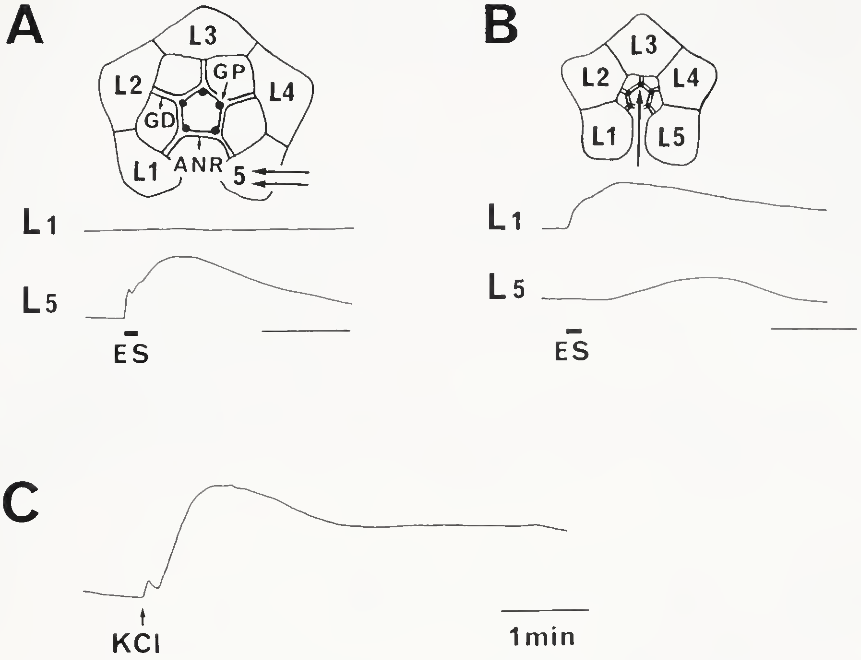

FIGURE 3. Contractions of gonad pretreated for 15 minutes with 0.5 *M* $MgCl_2$. Insets in A and B schematically show location of electrodes (thick arrows) viewed from inside. ANR: aboral nerve ring. GD: gonoduct. GP: genital pore. B: Stimulation given through the aboral region (arrow) of the test induced arrhythmic contractions in both L1 and L5 lobes. C: Arrhythmic contraction in response to addition of a few drops of 0.5 *M* KCl into the test. Rising and falling phases are slow.

responses to electrical stimulation of the aboral region (Fig. 3B) as well as to KCl application (Fig. 3C) were similar to that of the directly stimulated lobe and rhythmic movements were not initiated in any of the lobes tested.

To determine the site of the rhythm generator(s), all five lobes were first separated by cutting the lateral interconnections. Rhythmic contractions occurred normally in response to electrical stimulation of the aboral region. Separation of the lobe(s) from the test with each of the gonoduct(s) left intact did not abolish the rhythm. A preparation containing one lobe that was attached, via a gonoduct, to a small piece of the test with periproct, retained the rhythmic contractions. The rhythmicity disappeared, however, when the gonoduct was cut (L2 in Fig. 4C). Other lobes with intact gonoducts responded rhythmically (L1 and L3 in Fig. 4C).

Synchronous rhythmic contractions among gonadal lobes

The aboral nerve ring (ANR, inset of Fig. 3A) is a candidate for the rhythm generator located proximal to the gonoduct. The preparations were altered surgically as shown in insets of Figure 4. The stimulant used was KCl solution applied locally to the aboral region.

Simultaneous recordings from any three lobes showed that the phases of rhythmic contractions and the relative magnitudes were strikingly synchronized (Fig. 4A). However, supernumerary or lack of contractions occurred on rare occasions during the course of the rhythm. The synchronization was only slightly impaired by a single cut

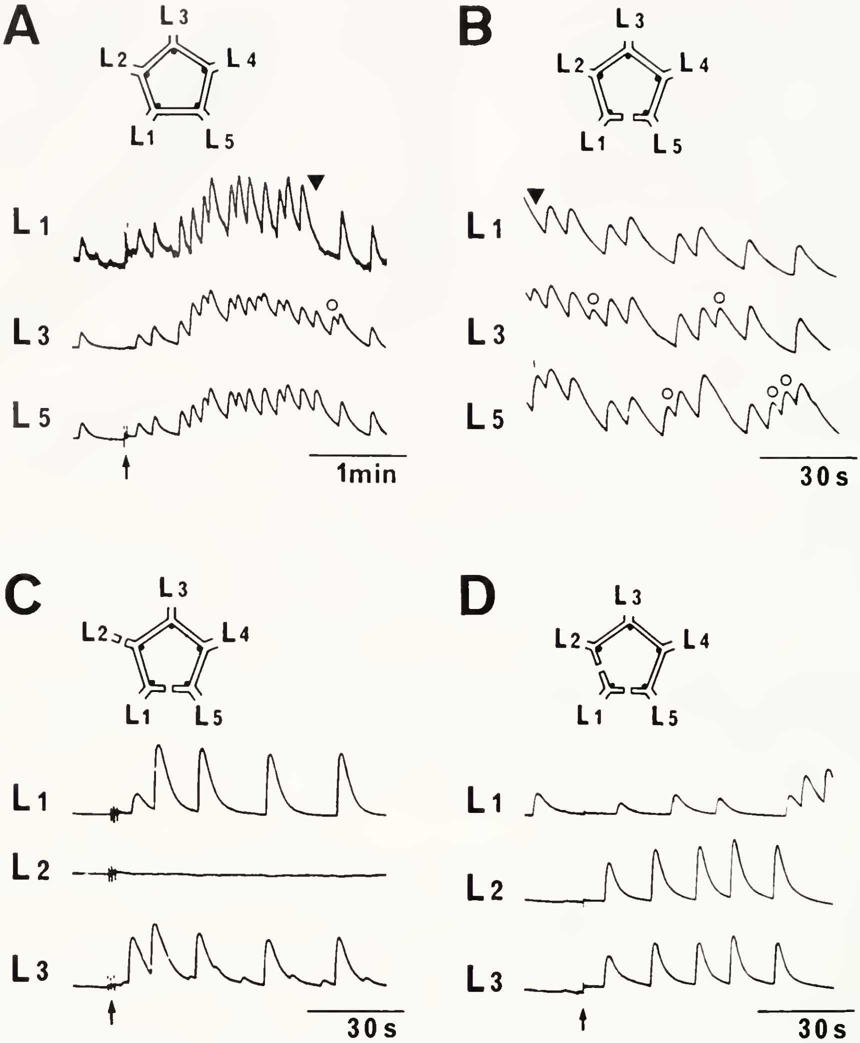

FIGURE 4. Simultaneous recordings from three lobes. A: ANR was intact. B: ANR was transected between L1 and L5. C: ANR was transected as in B with additional cut in the gonoduct of lobe L2. No contraction appeared in L2, but rhythmic contractions took place synchronously in L1 and L3. D: ANR was transected at two places, between L1 and L5 and between L1 and L2. L1 showed rhythmic contractions independently of the other two lobes. In A and B, triangles indicate lack of contraction in one lobe, while contractions took place synchronously in the other two lobes and circles indicate contractions occurring independently of the other lobes. Arrows indicate the time of KCl application.

in the ANR, although out-of-phase contractions tended to occur more frequently (Fig. 4B).

When the ANR was separated into two sectors, lobes in the same sector contracted synchronously but independently of those in the other sector (Fig. 4D). In most cases, the frequency of the rhythm was higher in the lobe connected to the larger sector.

When one out of the five gonoducts was cut transversely (Fig. 4C), the lobe (L2) with the severed gonoduct did not contract, while all other lobes contracted synchronously. The spread of the rhythm is, therefore, achieved by the coordinating information passing through the ANR and reaching the lobe via the gonoduct.

Conduction of excitation through ANR

Recordings from three lobes at high speed (10 mm/s) revealed a phase difference between onsets of the earliest and latest contractions, although they appeared to be occurring synchronously in slow speed recordings. The longest delay was observed when the ANR was cut at one place (Fig. 5B). As shown in the first group of contractions, L5, which was adjacent to the cut, started to contract earliest and the delay in onset proceeded in a sequence from L3 to L1. When a lobe opposite to the cut contracted earliest as in the second group of contractions (L3), the two lobes on either side of the cut (L1 and L5) contracted with a slight delay and almost at the same time. Even when the ANR was intact (Fig. 5A), the delay in onsets of contractions was evident, although only slight (the earliest and the latest being in L1 and L3 in the first group and those, in L1 and L5 in the second group, respectively).

The conduction velocity was calculated using the longest time lag between the

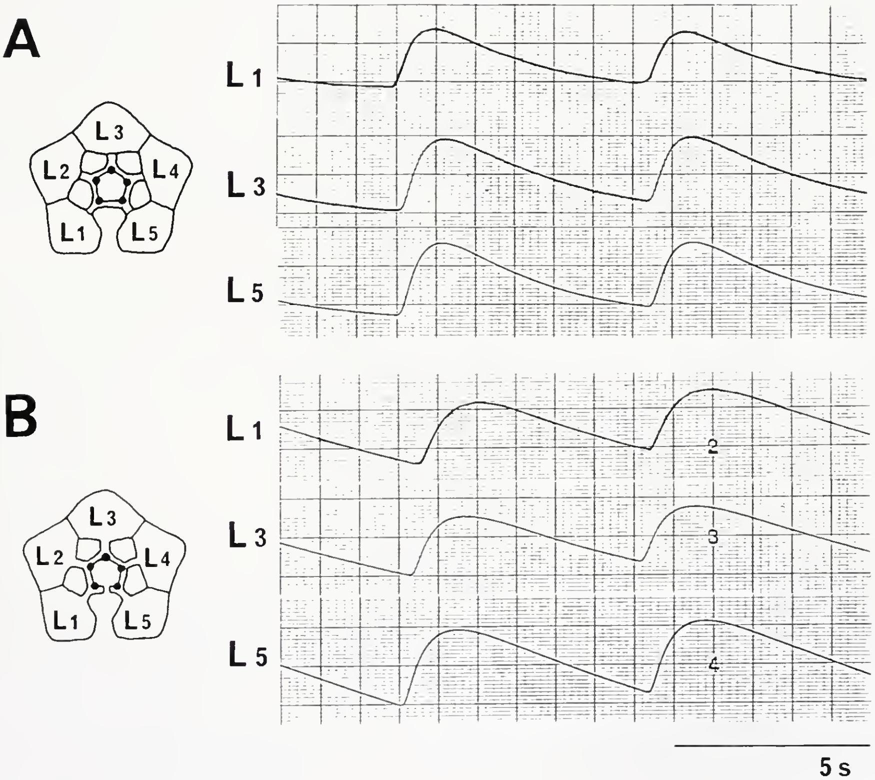

FIGURE 5. Simultaneous high speed recordings from three lobes. A and B were obtained from the same animal. In A, ANR was intact; in B it was cut as shown in the inset. A: Contractions started in sequence L1, L5, L3 at left and at right, L1, L3, L5. The time lag between onsets of the earliest and the latest contractions was 0.2 s in the left records and 0.3 s in the right. B: Contractions started in sequence at left L5, L3, L1 and at right L3, L5, L1. The time lags between onsets of the earliest and the latest contractions was 0.45 s in the left records and 0.25 s in the right.

onsets of the earliest and latest contractions and the distance between the genital pores belonging to the two contracting lobes. The time lag was 0.3 s and the distance, 9.2 mm, so that the velocity was 0.03 m/s at 28°C.

DISCUSSION

Rhythmic contraction

The present experiments demonstrate arrhythmic and rhythmic contractions of the gonad. The arrhythmic contractions were induced by electrical stimulation of the gonadal surface or ACh application. These responses were similar to those induced upon KCL application or electrical stimulation in the $MgCl_2$ pretreated gonad, suggesting that all the stimulants act directly on the gonadal muscle. The rhythmic contractions, apparently originate from neural activity since they were abolished by a transverse cut of the gonoduct, which presumably receives input from the ANR (Chuènot, 1948 cited from Smith, 1965). In fact, nerve bundles have been observed along the wall of the gonoduct using electron microscopy (pers. comm. from Yoshida).

The arrhythmic and rhythmic types differ in the time course of contraction. Gonad with gonoduct transected responded occasionally by a bi-phasic contraction to a brief electrical stimulation (Fig. 2B); the first phase being a single twitch-like contraction and the second phase, slower and of longer duration. The first phase resembles the rhythmic contractions, indicating a common neural mechanism. The second phase was similar to the arrhythmic contraction.

The fact that both the electrical stimulation and the KCl application evoked the arrhythmic contraction (direct stimulation of gonadal muscle) as well as the rhythmic contraction (indirect effect through neural elements) requires a modification of the conclusion (Iwata and Fukase, 1964b) which assumes only the direct effect of both stimulants on gonadal muscles.

The rhythm generator appears to reside outside the gonad in the ANR; information is conducted to the gonad via the gonoduct because preparations containing one gonadal lobe attached to a small piece of the aboral test retained the rhythmic contractions. Even when the ANR was cut at both sides adjacent to the gonoduct, the rhythmicity was not abolished, suggesting that the rhythm generator is located near the genital pore where the ANR connects with the gonoduct.

Synchronization of the rhythm

The rhythmic synchronous contractions among five gonadal lobes result in simultaneous gamete shedding. Each lobe has its own rhythm generator and when generators were connected by the ANR system, contraction occurred simultaneously in multiple lobes. However, either supernumerary or lack of contraction was seen in intact preparations and even more frequently in those with ANR transected at one place (Fig. 4B), suggesting that an interacting mechanism among the five generators helps to maintain the concerted rhythmicity.

Closer examinations revealed a time lag in onsets of contractions. It is assumed that the rhythm generator, which evokes the earliest contraction, acts like a pacemaker and also excites the other generators either directly or indirectly. The results indicate that the excitation from a pacemaker is conducted in both directions, with no one generator being dominant.

For each synchronous contraction, the maximum time lag between the earliest and the last contracting lobes was smaller when the ANR was intact (250 ms) than when it was severed at one place (450 ms). This difference may be due to the difference in spatial arrangements; the intact generators are arranged in a closed loop whereas, after severing, the arrangement is linear. Smith (1945) proposed a hypothetical model in the oral nerve ring for coordinative stepping of starfish tube feet. He assumed that each rhythm generator (motor center) innervates all the five radii. Such a scheme may not apply in the present case because the absence of contraction in the preparation with a single cut in the ANR was most frequently seen in the lobe furthest from the leading one. If the rhythm generator innervates the other four directly, then these lobes should have failed to contract. Perhaps each rhythm generator directly innervates only two adjacent generators, conducting the signals sequentially to the distal lobes.

The conduction velocity in the echinoderm nervous system has been measured by many workers: 0.04 m/s in *Echinus esculentus* (Cobb, 1968), *ca.* 0.07 m/s in *Arbacia lixula* (Millot and Okumura, 1968) and 0.14 to 0.20 m/s in *Strongylocentrotus franciscanus* (Sandeman, 1965). The conduction velocity measured by a quite different method in the present experiments gave about the same value. Our measured conduction velocity must be smaller than the true velocity for it was calculated from the delay between the earliest and the last contracted lobes, which included time required for conduction as well as the synaptic delay.

In the breeding season, the sea urchin gonads are filled with mature gametes and show rhythmic contractions of their lobes spontaneously as well as in response to artificial stimulations. When spent gonads were used, movements were only of a type of arrhythmic slow contraction. In the off season, gonads are filled with nutritive substances and gonadal lobes do not show rhythmic movements. A seasonal variation in internal conduction has been reported by Cochran and Englemann (1972) in *Strongylocentrotus purpuratus.* The concentration of spawning inducing factor in the radial nerves fluctuates annually, being highest during the breeding season. How this relates to rhythmic contraction is unknown.

ACKNOWLEDGMENTS

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REGULATION OF THE RELEASE OF CHROMATOPHOROTROPIC NEUROHORMONES FROM THE ISOLATED EYESTALK OF THE FIDDLER CRAB, *UCA PUGILATOR*

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ABSTRACT

Electrical stimulation of the isolated eyestalk of *Uca pugilator* induces the release of several peptides which affect epidermal chromatophores. Thresholds for release of these peptides were different, that for red pigment concentrating hormone (RPCH) being lowest, for black pigment dispersing hormone (BPDH) highest, and for black pigment concentrating hormone (BPCH) intermediate, but no red pigment dispersing hormone (RPDH) was detected at any voltage. Neurotransmitters, known to be present in crustacean central nervous systems, induced chromatophore dose dependent responses. Norepinephrine induced BPDH release, and dopamine induced both RPCH and BPCH release.

INTRODUCTION

Studies of the regulation of the release of peptide hormones from neuroendocrine cells have focused recently on the roles of neurotransmitters which inhibit or stimulate such release (Krulich, 1979; Moss, 1979). However, these studies have often resulted in conflicting observations due to dosage differences (Kordon *et al.,* 1981), feedback mechanisms (Krulich, 1979; Hökfelt *et al.,* 1980), or complex responses of the intact central nervous system (Fernlund *et al.,* 1980). Furthermore, simple implantation of high doses of potent neurotransmitters cannot distinguish between physiological and pharmacological responses (McKelvy *et al.,* 1980). For these reasons the trend has been to work with central nervous system fragments, hemisected pituitaries, isolated endocrine glands, or endocrine cells in culture (Krulich, 1979; Eiden and Brownstein, 1981; O'Donohue and Dorsa, 1982).

The endocrine control of pigment migration in chromatophores of crustaceans offers a sensitive tool for the investigation of neurotransmitters in endocrine regulation (Fingerman and Fingerman, 1977a, b). Peptidergic pigment dispersing and pigment concentrating chromatophorotropic hormones have been identified. A major site of synthesis of these peptides is the cell bodies of neuroendocrine cells in the medulla terminalis X-organ located within the eyestalk of most crustaceans. The axon terminals of these neuroendocrine cells comprise the sinus gland, the neurohemal organ, from which these neuroendocrines are released (Kleinholz and Keller, 1979; Cooke and Sullivan, 1983). Bioassays of these hormones can be performed in whole crabs, or isolated tissues, with both target cell specificity and picomolar sensitivity (Fingerman, 1973; Cooke *et al.,* 1977; Fingerman *et al.,* 1981a; Jaffe *et al.,* 1982; Riehm and Rao,

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Abbreviations: BPDH, black pigment dispersing hormone; BPCH, black pigment concentrating hormone; RPDH, red pigment dispersing hormone; RPCH, red pigment concentrating hormone; DA, dopamine; NE, norepinephrine; 5-HT, 5-hydroxytryptamine; BE, bretylium tosylate; S, saline; WRS, Wilcoxon rank sum test; mOS, milliosmoles; R_f, retention factor.

1982). Cooke *et al.* (1977) reported that the isolated sinus gland releases red pigment concentrating hormone (RPCH) in a voltage dependent, Ca^{++} sensitive manner. However, several other endocrines are also stored in the sinus gland and are probably released simultaneously with RPCH when such *in vitro* experiments are performed.

Rao and Fingerman (1970, 1975), Fingerman and Fingerman (1975; 1977a, b), Fingerman *et al.* (1981a, b), and Hanumante and Fingerman (1981a, b; 1982a, b) have reported that various neurotransmitters injected into fiddler crabs can induce specific endocrine mediated chromatophore responses. These authors suggested that the neurotransmitters trigger the release of specific chromatophorotropic hormones. The neurotransmitters have no effect when applied directly to the chromatophores in isolated legs. Briefly, these investigators found that norepinephrine (NE) indirectly induces black pigment dispersion, 5-hydroxytryptamine indirectly induces red pigment dispersion, and dopamine (DA) indirectly induces red pigment concentration. These observations are the only substantial evidence that neurotransmitters affect the release of arthropod neuroendocrines, despite a large literature on insect endocrinology (Gilbert *et al.,* 1980; Riddiford, 1980; Orchard and Loughton, 1981; Orchard *et al.,* 1982).

We designed experiments to learn whether the neurotransmitters NE and DA can affect endocrine release from the isolated eyestalk complex *in vitro.* In addition, the composition and biological activity of perfusates of isolated eyestalks which were electrically stimulated were determined.

MATERIALS AND METHODS

Fiddler crabs, *Uca pugilator,* were obtained from the Gulf Specimen Supply Company, Panacea, Florida, and kept in a closed circulating sea water system until use (22°C; LD 12:12), lights on at 0800 hours. Eyestalks from crabs (15–18 mm carapace width) were dissected in saline (Cooke *et al.,* 1977) with the aid of a microscope. The entire optic tract including the major ganglia and sinus gland were removed from the eyestalk. These tissues were maintained in a 50 μl drop of saline that was periodically replaced, with the aid of a microliter syringe. The eyestalk was held in place with a suction electrode attached to the cut stump of the optic nerve. Electrical stimulation was given via the suction electrode in 40 ms duration pulses of various voltages (0.1, 0.5, 5, 10, 15, 20 volts). The order was randomized so the eyestalk did not receive a simple increasing stimulation. After a stimulation bout (2 minutes continuous voltage, 3 minutes rest) the saline perfusing the eyestalk was removed with a syringe and retained for bioassay. Voltage was delivered with a Phipps and Bird model 511 stimulator. Saline containing either a drug or neurotransmitter perfused the eyestalk for 5 minutes. The fluid was then removed with a syringe and retained for bioassay. The various concentrations of drugs or neurotransmitters were also presented in a random order. If both chemical and electrical stimulation were required the eyestalk was first perfused with the given drug or neurotransmitter for 5 minutes. Electrical stimulation was delivered to the tissue still in the saline containing drug or neurotransmitter. After the 3 minute rest the saline was removed for bioassay. The eyestalk was washed with fresh saline for 5 minutes between each electrical or chemical stimulation. An individual eyestalk was maintained *in vitro* for no more than two hours.

Perfusates were kept at 4°C until bioassayed. *Uca pugilator* exhibits a circadian rhythm of chromatophoric pigment dispersion and concentration. The pigments are more dispersed by day than at night. All eyestalks were dissected and stimulated between 0900 and 1130 hours. This ensured that some pigment dispersing endocrines were still stored in the sinus gland, which are reduced and replenished daily as a consequence of this rhythm (Fingerman and Fingerman, 1977b). All bioassays using isolated legs were performed in the afternoon with legs from either eyestalk ablated crabs or crabs adapted to a black background (Herman and Dallmann, 1975; Hanumante and Fingerman, 1981a, b; Quackenbush, 1981). Eyestalkless crabs have maximally concentrated black and red pigments and provided the legs for the bioassay for pigment dispersing hormones. Intact crabs adapted to a black background have maximally dispersed black and red pigments and were used in bioassays for pigment concentrating hormones. An aliquot of each perfusate was carefully injected into 3 isolated legs (5 μl/leg) to avoid tissue damage. The black and red chromatophores were staged using the method of Hogben and Slome (1931) wherein stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions. The chromatophores were staged at the time of injection and 15, 30, 45, and 60 minutes thereafter. Isolated legs remain responsive to physiological doses of hormone for at least 60 minutes after removal from the crab (Herman and Dallmann, 1975; Quackenbush, 1981). Activity of the perfusates was determined using a modification of the Standard Integrated Response (SIR) technique of Fingerman *et al.* (1967). The activity values presented herein were calculated as follows. In assays for pigment dispersing actvity the sum all of the chromatophore stages determined at 0, 15, 30, 45, and 60 minutes with three legs given a control perfusate was subtracted from the sum of all the corresponding stages for three legs injected with perfusate from a stimulated eyestalk. The difference is the activity value. For example, if no change occurs in controls (3, +3 +3 +3 +3 = 15) and a maximum dispersion occurs in experimentals (3, +15 +15 +15 +15 = 63) the activity would be 48 (63 − 15), a maximal, possible response. On the other hand, to determine pigment concentrating activity the sum of the stages of isolated legs given a perfusate from a stimulated eyestalk was subtracted from the sum for the isolated legs given a control perfusate. Thus activity is the difference between the responses of the experimental and control legs. The activity values from the replicate experiments were averaged and standard deviations were calculated. Chromatophore stages are quantal data that cannot fulfill the requirements for parametric statistical analysis. Therefore, the conventional nonparametric substitute for the standard *t*-test, the Wilcoxon Rank Sum (WRS), was used to test differences between treatments and controls (Sokal and Rohlf, 1969).

The drugs were dissolved in isosmotic saline, 850 mOS, which had been aerated for one half hour prior to use. These solutions were prepared initially at 10^{-4} M and then serially diluted to achieve the desired doses. All drug solutions were prepared daily from dry frozen stocks. Norepinephrine hydrochloride and dopamine hydrochloride were purchased from Sigma Chemical Corp. Bretylium tosylate (Lot 177-36) was a gift of American Critical Care. Spiperone (lot A3301) was a gift of Janssen Pharmaceutica. In one set of experiments, saline with spiperone (10^{-5} M or 10^{-4} M) was injected into intact crabs (0.05 ml/crab). The crabs were then transferred from a black pan to a white pan in order to induce pigment concentration. This experiment tested whether spiperone, a dopamine antagonist, could block pigment concentration in intact crabs.

A small column of Sephadex G-25 was equilibrated with 50 mM phosphate buffer (pH 7.2). The void volume of the column (225 μl) was determined with blue dextran. The column was calibrated with Cytochrome C (12,400 d) and Bacitracin (1411 d). Perfusates from a single isolated eyestalk stimulated with 10 volts were pooled, lyophilized, then resuspended in 50 μl of the phosphate buffer (pH 7.2), applied to the top of the column, and eluted with the same buffer. Fractions of 25 μl were collected, adjusted to 850 mOS with 400% saline (Cooke *et al.*, 1977), and then bioassayed. The retention factor, R_f, was calculated by the formula: Void volume ÷ Elution volume. Protein was determined by the method of Peterson (1977). Synthetic RPCH was purchased from Peninsula Laboratory (Belmont, California) and run on the Sephadex column as a standard.

RESULTS

Isolated eyestalks electrically stimulated

Eyestalks stimulated with 10 volts or more released substances which induced significant black pigment dispersion in isolated crab legs ($P < 0.05$, WRS test) (Fig. 1). No significant red pigment dispersing activity was detected after voltage stimulation (0.1 to 20 volts). Perfusates of eyestalks stimulated at 15 volts had significantly more black pigment dispersing hormone (BPDH) activity than those given 10 volts ($P < 0.05$, WRS test). Black pigment concentrating hormone (BPCH) activity was detected in perfusates of eyestalks given 0.5 volt; at 5 volts maximum BPCH activity was observed ($P < 0.05$, WRS test). RPCH activity was found in perfusates of eyestalks stimulated with 0.1 volt ($P < 0.05$, WRS test). The apparently different thresholds for the release of BPDH and BPCH may have been due to the antagonistic actions of these two peptides on the target cells. Perfusates of eyestalks stimulated with 5 volts had no significant BPDH activity (Fig. 2). However, perfusates from eyestalks stimulated with 10 volts had significant BPDH activity and the BPCH activity was clearly antagonized by BPDH, as seen by the drop of BPCH activity in Figure 1, and Figure 2. RPCH activity was not antagonized by red pigment dispersing hormone (RPDH) in perfusates from eyestalks stimulated with 5 or 10 volts (Fig. 3). Either RPCH completely antagonizes the action of any RPDH that might have been released, or no RPDH was released with this stimulation.

The data presented in Figure 1 demonstrate that the eyestalk releases several different factors when stimulated with 10 volts with this protocol. The isolated eyestalk may release only totipotent hormones which affect all chromatophores equally, as

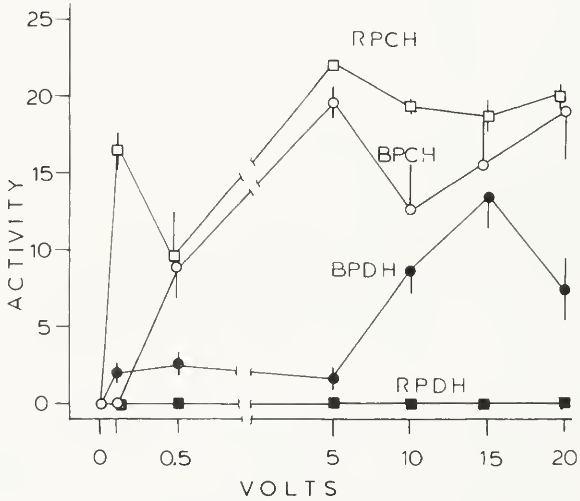

FIGURE 1. Activity of perfusates from isolated eyestalks stimulated with various voltages *in vitro*. RPDH activity (filled squares), RPCH activity (open squares), BPCH activity (open circles), and BPDH activity (filled circles) are compared to saline controls from unstimulated perfusates. Each point is the mean ± one standard deviation for 21 isolated legs given stimulated perfusate compared to 21 isolated legs given control perfusate (seven replicates).

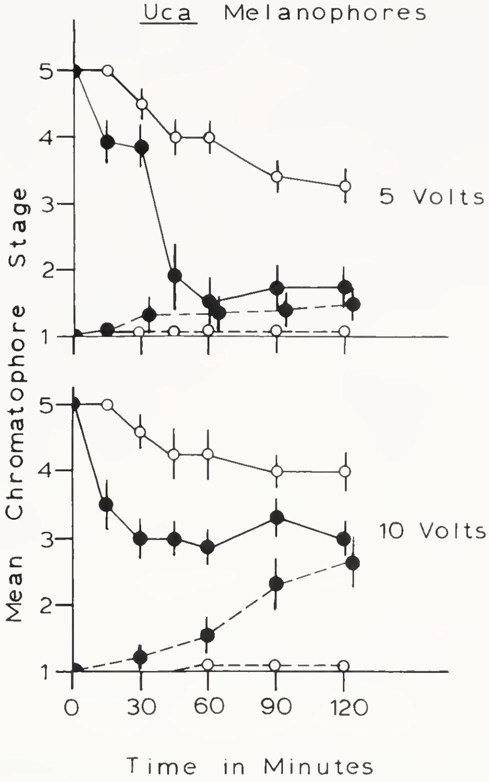

FIGURE 2. Mean chromatophore stages of isolated legs given perfusates from isolated eyestalks stimulated with 5 volts or 10 volts (solid circles) compared to isolated legs given perfusates from unstimulated isolated eyestalks (open circles). Solid lines connect the values for isolated legs with initially fully dispersed black pigment. Dashed lines connect the values for isolated legs with initially fully concentrated black pigment. All values are means ± one standard devation ($n = 21$, seven replicates).

proposed by Josefsson (1983), or the eyestalk may release hormones specific for each type of chromatophore. One way to distinguish among these alternatives would be to separate the chromatophorotropins in the perfusates. Therefore we chromatographed pooled perfusates from 10 volt stimulation bouts to determine if totipotent peptides or peptides with target cell specificity were being released. Assays were performed for BPDH, BPCH, RPCH, and RPDH activities in each fraction after the void volume.

Red pigment concentrating activity was found in 4 different fractions (R_f mean ± one standard deviation for 4 replicates; 0.83 ± 0.02; 0.65 ± 0.03; 0.50 ± 0.05; and 0.34 ± 0.02) (Fig. 4A). The fraction at R_f 0.50 had 0.9 ± 0.3 pg protein, and co-chromatographed with BPDH activity. This fraction (0.50 ± 0.02) had no BPCH activity. The fraction at R_f 0.34 contained a significant amount of RPCH activity ($P < 0.05$, WRS test) which co-chromatographed with the synthetic RPCH. The *Uca* material at R_f 0.34 had no other activity in these assays. The fraction at R_f 0.625 had both BPCH and RPCH activity, at slightly different levels. RPDH activity was not detected in any fractions assayed. We see that stimulation of the isolated *Uca* eyestalk results in the release of several different compounds, some sharing biological activity while others induce a specific target cell response.

Substances with BPCH activity were found in three fractions (R_f 0.63 ± 0.02;

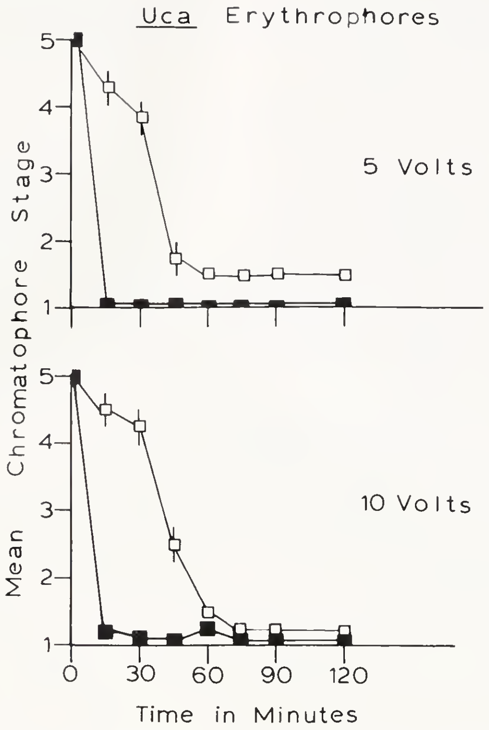

FIGURE 3. Mean chromatophore stage of isolated legs given perfusates from isolated eyestalks stimulated with 5 volts or 10 volts (solid squares) compared to isolated legs given perfusates from unstimulated eyestalks (open squares). Solid lines connect the values from isolated legs with fully dispersed red pigment. Isolated legs with initially fully concentrated red pigment were also tested, but showed no effect of perfusate injection (data not shown). All values are means ± one standard deviation (n = 21, seven replicates).

0.43 ± 0.03; and 0.38 ± 0.01). Two different fractions had peptides (0.6 ± 0.02 pg protein for each fraction) in the low molecular weight range (less than 5,000 d) that only affected black chromatophores. BPDH activity was resolved into 3 fractions after the void volume (R_f 0.50 ± 0.02 had 0.9 ± 0.3 pg protein, 0.75 ± 0.02 and 0.41 ± 0.01 each had 0.4 ± 0.15 pg protein). It should be noted that two fractions containing BPDH had no BPCH activity. The presence of several sizes of peptides which have biological activity in these perfusates suggests that processing of precursors may occur within the eyestalk complex. Furthermore, the isolated eyestalk complex releases these large sized compounds when stimulated with voltage. Finally, the eyestalk complex contains large compounds which can affect two different target cells, as well as different compounds which have activity on only one target cell.

Isolated eyestalks stimulated with NE

Isolated eyestalks were bathed in saline to which one of six different NE concentrations was added (10^{-11}–10^{-6} *M*) (Fig. 5). Exogenous NE did not induce the release of any substance which produced either significant dispersion or concentration of the red pigment. Isolated eyestalks in saline with 10^{-11} *M* NE released BPDH in detectable

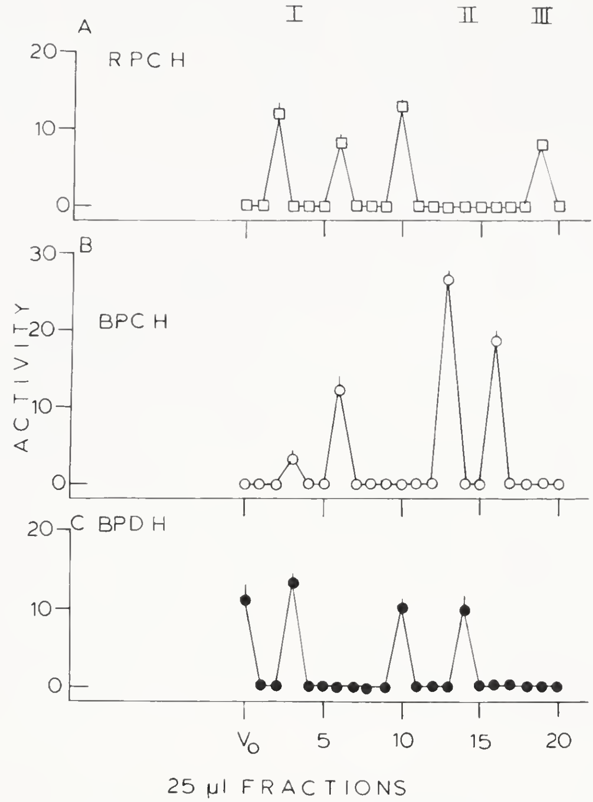

FIGURE 4. Activity of fractions eluted from a Sephadex G-25 column of the perfusate of isolated eyestalks (n = 4) stimulated with 10 volts on isolated legs. RPCH (panel A) activity is indicated by open squares. BPCH (panel B) activity is indicated by open circles. BPDH (panel C) activity is indicated by solid circles. Each activity value is the mean ± one standard deviation for 12 legs (four replicates). Numeral I indicates where Cytochrome C (12,400 d) eluted; II indicates where Bacitracin (1,411 d) eluted; and III where synthetic RPCH eluted (n = 12, four replicates).

quantities ($P < 0.01$, WRS test). The dose response curve (Fig. 5) of the effect of exogenous NE on isolated eyestalks reveals that the most BPDH was released in response to 10^{-9} *M* NE. Increasing the NE concentration above 10^{-9} *M* significantly decreased the level of BPDH in the perfusates (WRS test $P < 0.05$).

In a separate series of experiments isolated eyestalks were perfused with 10^{-6} *M* NE, then stimulated with 10 volts. Stimulation with both 10^{-6} *M* NE and 10 volts resulted in significantly more BPDH in the perfusate than stimulation with 10^{-6} *M* NE alone or 10 volts alone ($P < 0.01$, WRS test, Fig. 6). As expected, no significant red pigment dispersion was detected in perfusates from any combination of stimulation with volts and NE. Isolated eyestalks given bretylium (2×10^{-4} *M*), a blocker of impulse mediated NE release (Boura and Green, 1959), and subsequently stimulated with 10 volts released no detectable BPDH into the perfusate (Fig. 6). Since the chromatophores on isolated legs are unaffected by NE or bretylium (Fingerman *et al.*, 1981a, b; Hanumante and Fingerman, 1981a, b; 1982a, b) we conclude that NE can induce the release of BPDH from the isolated eyestalk.

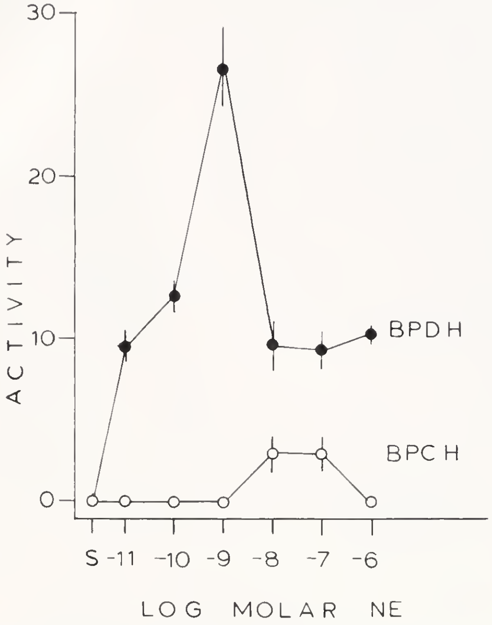

FIGURE 5. Dose response curve for the effect of NE on the biological activity of the perfusate of isolated eyestalks in isolated legs. BPDH activity is represented by solid circles and BPCH activity is represented by open circles. Each value is the mean ± one standard devation (n = 27, nine replicates). Saline value indicated by S.

Isolated eyestalks stimulated with DA

DA produces red pigment concentration when injected into whole crabs, but it has no effect on the chromatophores of isolated legs (Fingerman and Fingerman,

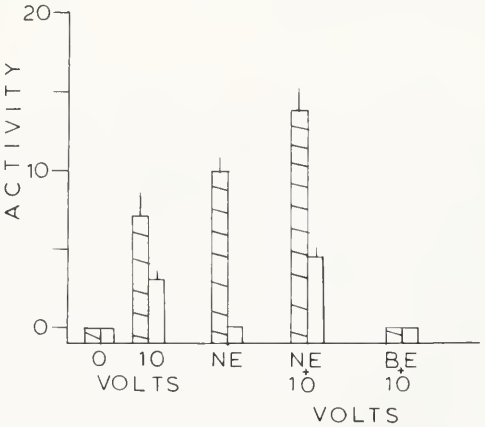

FIGURE 6. The effect of NE (10^{-6} M) and voltage stimulation (10 volts) on BPDH and RPDH activity in perfusates from isolated eyestalks. Open bars are values for red pigment and hatched bars are values for black pigment dispersion in isolated legs given perfusates from isolated eyestalks treated as indicated. Bretylium (2×10^{-4} M) was added to saline which perfused eyestalks prior to voltage (10 volt) stimulation indicated by BE + 10. All values are means ± one standard deviation (n = 18, six replicates).

1977a; Fingerman *et al.*, 1981a). This exogenous DA presumably exerts its effect by triggering RPCH release. The site of action of the DA could be the eyestalk or the central nervous organs within the body proper such as the supraesophageal ganglia. The crustacean central nervous system within the body proper not only contains RPCH (Brown, 1973) but also DA (Eloffson *et al.*, 1982).

Isolated eyestalks were bathed in 8 concentrations of DA (10^{-12}–10^{-5} *M*). At all but the weakest dose significant, amounts of RPCH and BPCH were detected in the bathing media (Fig. 7) ($P < 0.05$, WRS test). Although DA did not induce black pigment concentration *in vivo* (Fingerman and Fingerman, 1977a), significantly more BPCH than RPCH was found in the perfusates (WRS $P < 0.01$, 10^{-10}, 10^{-9}, 10^{-6}) when tested *in vitro*. DA has no direct action on either type of chromatophore when injected into isolated legs.

In order to add support to the new observation that DA can mediate the release of BPCH, the well known mammalian DA antagonist, spiperone, was used in *in vivo* and *in vitro* experiments. In intact whole crabs given 10^{-5} or 10^{-4} *M* spiperone, concentration of the black pigment was blocked but red pigment concentration was unaffected (Fig. 8). When isolated *Uca* eyestalks were stimulated with 5 volts the perfusate contained both RPCH and BPCH (Fig. 1). We then used a 5 volt stimulation of isolated eyestalks bathed in saline with spiperone to determine the effect *in vitro*. Perfusates from isolated eyestalks stimulated with 5 volts in saline with spiperone at 10^{-9} *M* had no BPCH but did have RPCH (Fig. 9). At higher concentrations of spiperone only BPCH was absent from the perfusate. Spiperone may act on the

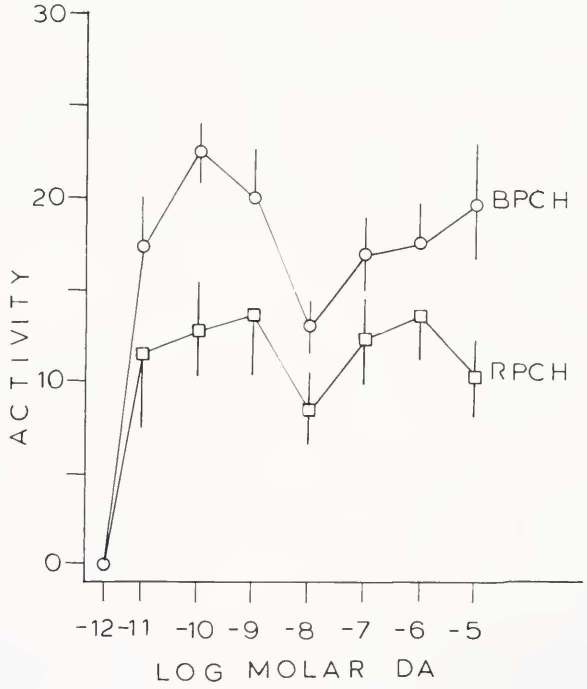

FIGURE 7. Dose response curve for the effect of DA on the biological activity of the perfusate of isolated eyestalks. The assays were performed on isolated legs. RPCH activity is represented by open squares and BPCH activity is represented by open circles. Each value is the mean ± one standard deviation (n = 27, nine replicates).

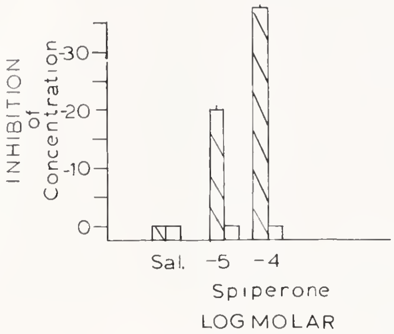

FIGURE 8. The effect of spiperone on pigment concentration in intact crabs which were changed from a black background to a white background. Open bars are values for red pigment and hatched bars are values for black pigment. Control crabs concentrate their black and red pigments when this background change experiment is performed. Activity was calculated as described in Methods, negative values indicate inhibition. For this experiment activity values were based on 5 crabs given treatments compared to 5 crabs as control. Saline values are indicated by Sal, spiperone in saline at 10^{-5} *M* values by 5 and spiperone in saline at 10^{-4} *M* values by 4. All values are means ± one standard deviation (n = 30 each group, six replicates).

eyestalk to produce extraordinarily large BPDH release, so an additional experiment was done. Eyestalks were bathed in saline containing spiperone then stimulated with 10 volts. This perfusate was then passed through the small Sephadex column and BPDH, RPCH, and BPCH bioassays were done on each fraction (Table I). BPDH activity was not affected by spiperone. Perfusates with spiperone had significantly more of the smallest RPCH than the control perfusates though no effect on the RPCH's of two other sizes were detected. Spiperone at 10^{-6} *M* completely blocked

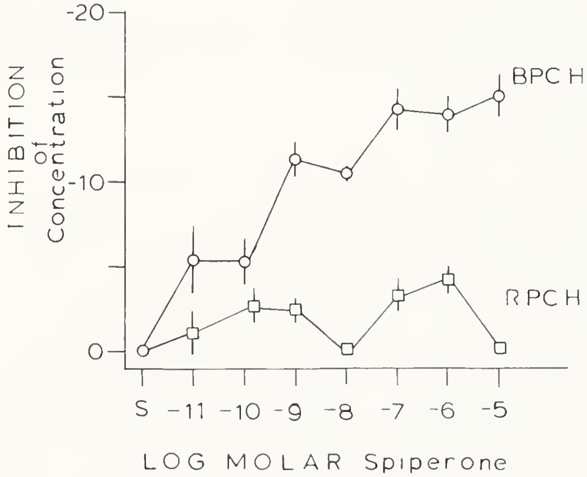

FIGURE 9. Dose response curve for the effect of spiperone in saline bathing isolated eyestalks stimulated with 5 volts on the presence of BPCH and RPCH measured in isolated legs. BPCH (open circles) and RPCH (open squares) activity was determined as described, negative values indicate inhibition. Increasing values mean increasing inhibition of BPCH or RPCH release as compared to controls of saline perfused eyestalks stimulated with 5 volts. Each value is the mean ± one standard deviation (n = 27, nine replicates). Saline bathing eyestalks stimulated with 5 volts (control) indicated by S.

the voltage induced release of all forms of the BPCH expected with a 10 volt stimulation (Table I).

DISCUSSION

The control of pigment migration in integumental chromatophores of the fiddler crab *Uca pugilator* by antagonistic peptide hormones presents a difficult challenge to the neuroendocrine system. The hormones we studied are all produced in and released from the same neurohemal complex within the eyestalk. However, crabs can maintain the pigment in each type of chromatophore in different stage of dispersion so the black pigment may be fully dispersed while the red pigment is fully concentrated. Therefore, some mechanism for the independent release of specific chromatophorotropic hormones may exist.

Stimulation of the isolated *Uca* eyestalk with different voltages apparently differentially releases RPCH, BPCH, and BPDH. This stimulation may act upon neurosecretory cells directly or on neurons presynaptic to the neurosecretory cells. The apparently high threshold for BPDH release may be a result of the presence of its antagonist BPCH. Similarly, the low threshold of RPCH release may mask the presence of RPDH which was not detected in any of our experiments. Thus regulation of chromatophore stage in crabs could be the result of different levels of the antagonistic pairs of hormones acting on the target cells (Rao and Fingerman, 1983). This is supported by the comparison of biological activities of the components of the pooled 10 volt perfusate before and after separation by gel filtration chromatography (Figs. 1 and 4). The total BPCH activity increased after separation from its antagonist, BPDH. The increase of total activity of the RPCH after chromatography also argues for the presence of RPDH, though it was not detected.

Electrical stimulation of the isolated eyestalk triggers release of peptides which only affect one target cell (Fig. 4). This contradicts the observation that crustaceans use peptides which are totipotent (affecting both melanophores and erythrophores) as the primary regulators of color change (Fernlund and Josefsson, 1972; Quackenbush, 1981; Josefsson, 1983). Previous results using catecholamines and 5-HT to trigger release of chromatophorotropins suggested each neurotransmitter had a specific effect that could not be a result of the release of a totipotent peptide (Rao and Fingerman,

TABLE I

Effect of spiperone (10^{-6} M) on voltage stimulated release of BPDH, RPCH, and BPCH activity from isolated eyestalks

		Activity (mean ± one standard deviation)		
	R_f	10 volts	10 volts + 10^{-6} *M* spiperone	Significance
BPDH	0.76	13.55 ± 1.1	12.55 ± 0.89	NS
	0.50	10.36 ± 1.5	10.1 ± 1.1	NS
RPCH	0.625	8.8 ± 0.89	10.3 ± 1.7	NS
	0.50	13.2 ± 0.68	13.0 ± 0.68	NS
	0.34	8.2 ± 0.73	23.0 ± 1.18	$P < 0.01$
BPCH	0.625	12.5 ± 1.5	0	$P < 0.001$
	0.43	26.0 ± 1.09	0	$P < 0.001$
	0.38	18.5 ± 1.10	0	$P < 0.001$

1970; Fingerman and Fingerman, 1975; 1977a, b; Fingerman *et al.,* 1981a, b). Those experiments with intact crabs did report that the brain was one site for the action of the neurotransmitter, though the eyestalk was also a likely target. The present *in vitro* study confirmed *in vivo* studies which demonstrated that NE only induces BPDH release (Fingerman and Fingerman, 1977; Fingerman *et al.,* 1981). Bretylium blocked voltage induced release of BPDH, which suggests that voltage stimulation acted through a pathway involving NE release. High (10^{-6} M) levels of NE released significantly less BPDH than lower doses (10^{-9} M). This has been observed previously with neurotransmitter induced release of vertebrate peptides (Koch, 1970; Bower *et al.,* 1974; Hadley *et al.,* 1975). A negative feedback role for the neurotransmitter was postulated. Hanumante and Fingerman (1982b) have already postulated a negative feedback mechanism for NE regulation of BPDH based on pharmacological experiments *in vivo.* The observation here that voltage stimulation can augment 10^{-6} M NE induced BPDH release provides additional support for this hypothesis.

Fingerman and Fingerman (1977a) found 2.6×10^{-3} M DA induced RPCH but not BPCH release in whole crabs maintained on a black background. In contrast, BPCH release by DA was apparent in experiments with isolated eyestalks and isolated legs (Fig. 7). The crabs were presumably releasing BPDH into their blood to keep the black pigment dispersed in response to that background. In intact crabs circulating BPDH could have antagonized and masked any released BPCH. In the *in vitro* experiments there was presumably insufficient BPDH to mask all of the BPCH in the injected perfusate. We now find that dopamine can induce both RPCH and BPCH release *in vitro.* However, spiperone, in both *in vivo* and *in vitro* assays only affects the release of BPCH, not RPCH. Clearly, there must be a further subdivision of DA stimulation of peptide release in the isolated eyestalk, perhaps similar to the mammalian dopamine_1 and dopamine_2 systems (Lichtensteiger, 1979).

The actual mechanisms by which the crustacean sinus gland controls the release of specific hormones is not known. This neurohemal organ stores several distinct hormones, and some produce antagonistic actions in the crab (*e.g.,* BPDH and BPCH). The voltage dose response curves (Fig. 1) suggest such differential thresholds for endocrine release may provide an experimental approach for studying the release of target cell specific hormones. A physiological mechanism may be the specific release of a neurotransmitter inducing release of a specific hormone from a peptidergic neuron. Clearly NE has a singular action, inducing BPDH release.

The demonstration here and by others (Cooke *et al.,* 1977; Newcomb, 1982) that the crustacean eyestalk neuroendocrine system releases several peptides of different sizes suggests that these peptides are processed from larger precursors. The demonstration of large and small peptides with the same biological activities (Fig. 4) supports the notion that processing into smaller peptides can occur at the sinus gland.

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EFFECTS OF AMINO ACIDS, MAGNESIUM, AND MOLLUSCAN EXTRAPALLIAL FLUID ON CRYSTALLIZATION OF CALCIUM CARBONATE: *IN VITRO* EXPERIMENTS

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ABSTRACT

The extrapallial fluid (EPF), the fluid component of the shell-forming system of molluscs, has been examined for its effect on the rate of $CaCO_3$ crystal formation *in vitro*. The medium was an artificial inorganic extrapallial fluid supersaturated with respect to $CaCO_3$. EPF of the bivalves *Crassostrea virginica* and *Mercenaria mercenaria* strongly inhibited $CaCO_3$ crystallization in high dilution. The inhibitory material had negatively charged groups as indicated by the removal of inhibition subsequent to passage through a DEAE-Sephadex column and the restoration of inhibition in material eluted from the column with 2.0 *M* NaCl. Polyaspartic and polyglutamic acids with COO^- groups also inhibited crystal formation whereas polyamino acids lacking those groups and free amino acids were without effect. The inhibition by the acidic polyamino acids suggests that polypeptides known to be present in EPF are one possible cause of inhibition of crystal formation by EPF. Mg present in EPF was also strongly inhibitory. The observed inhibition of *in vitro* crystallization by EPF and Mg indicates that these substances could be factors in governing $CaCO_3$ deposition rate in molluscan shell.

INTRODUCTION

A wide range of studies has been devoted to the effects of inorganic ions and organic compounds on the rates of formation of crystals of calcium carbonate and calcium phosphate. These varied studies have attempted to gain insight on the mechanisms controlling crystal formation in natural waters, in invertebrates and vertebrates, and in marine algae.

In molluscs and marine algae, which have calcium carbonate skeletons, the organic compounds of the skeletons have been given attention because of their possible roles in crystal nucleation (Crenshaw, 1972a; Weiner and Hood, 1975; Crenshaw and Ristedt, 1976; Weiner, 1979; Samata *et al.*, 1980; Weiner and Traub, 1981), inhibition of crystal growth (Crenshaw and Ristedt, 1976; Wilbur, 1976; Weiner, 1979; Weiner and Traub, 1981), and crystal orientation (Weiner and Traub, 1981). By means of an *in vitro* method, inhibition of $CaCO_3$ crystal formation has been demonstrated for the organic material in sea water (Chave and Suess, 1967, 1970), for the soluble organic matrix of the shell of the oyster *Crassostrea virginica* (Wheeler *et al.*, 1981); and for the polysaccharide associated with coccoliths of the alga *Emiliania huxleyi* (Borman *et al.*, 1982). However, nucleation by individual invertebrate proteins has not been demonstrated. In addition to investigations on calcium carbonate crystallization, a number of compounds and conditions have been studied for their effects on the rate of *in vitro* calcium phosphate crystallization (Boulet and Marier, 1961; Bachra and Fisher, 1969; Francis *et al.*, 1969; West and Storey, 1972; Robertson,

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AIEF, artificial inorganic extrapallial fluid; EFP, extrapallial fluid.

1973; Termine and Eanes, 1974; Termine and Conn, 1976; Moreno *et al.*, 1979; Boskey and Posner, 1980; Termine *et al.*, 1980).

In molluscan shell, the calcium carbonate crystals are formed on the inner shell surface in association with the extrapallial fluid and an organic matrix, both derived from secretion of the mantle epithelium. The extrapallial fluid contains inorganic ions, including calcium and magnesium, and various organic compounds, including proteins, carbohydrates, and amino acids (see Wilbur and Saleuddin, 1983). The initiation and inhibition of $CaCO_3$ crystallization in molluscs, have been attributed to the polypeptide and carbohydrate moieties of the organic matrix. Weiner and Traub (1981) hypothesized that Ca-binding COO^- groups of polypeptide chains may initiate or inhibit crystal growth, depending upon their spacing relative to the spacing of Ca of the $CaCO_3$ crystal lattice. Crenshaw and Ristedt (1976) have suggested that sulfated polysaccharide groups which bind calcium may initiate $CaCO_3$ crystal nucleation by the process of ionotropy. However, if the material bearing the sulfated groups is secreted by the mantle on growing crystals, the sulfated groups are thought to attach to calcium on the crystal surface lattice, thereby inhibiting further additions of ions and halting crystal growth.

In the present study, two sets of experiments are reported. The first set examines the influence of the extrapallial fluid in initiating or inhibiting $CaCO_3$ crystallization *in vitro*. Magnesium, a component of extrapallial fluid and an inhibitor of $CaCO_3$ crystallization in sea water (Pytkowicz, 1965) has also been studied for its effect on crystallization rate. The second set of experiments evaluates the effects of charges associated with organic molecules on $CaCO_3$ crystallization rates. Extrapallial fluid from which negatively charged substances were removed and free and polyamino acids differing in charge have been studied. A solution containing the major inorganic components of molluscan extrapallial fluid was used as a test system.

The experiments on free and polyamino acids were undertaken in collaboration with Dr. C. S. Sikes.

MATERIALS AND METHODS

Media

Artificial inorganic extrapallial fluid (AIEF) was prepared based on analyses of the inorganic components of molluscan extrapallial fluid (Crenshaw, 1972b; Wada and Fujinuki, 1976). Ionic concentrations are assembled in Table I. All reagents used in AIEF were analytical grade with the exception of highly pure $CaCl_2$ obtained from Sigma Chemicals. Solutions were prepared weekly and kept at 4°C.

Crystallization assay

Thirty ml of AIEF were placed in a 100-ml round bottom flask. One ml of the test substance in glass distilled water or 1.0 ml glass distilled water (control) was added and the flask was stirred by means of a magnetic stirrer and a 3×1.5 mm teflon stirring bar. A pH electrode secured in the flask by a well-fitting rubber collar was immersed in the fluid. The pH of the solution was adjusted to 8.60 with NaOH to offset the pH decrease that occurs when the $CaCl_2$ is added. The flask was tightly stoppered to prevent exchange with outside air, and monitored by means of a chart recorder connected to an expanded scale pH meter (Fisher Accumet 107). The pH was recorded continuously beginning with the addition of 0.9 ml of 1.0 *M* $CaCl_2$ which supersaturated the solution and lowered the pH to 8.33. The final Ca concentration was 28.21 m*M*. During most experiments, the temperature was kept at

TABLE I

Inorganic components of normal and experimental extrapallial fluids

ion	analysis 1*	analysis 2**	our AIEF***
	m*M*	m*M*	m*M*
Na^+	441–444	422.8–431.5	442
K^+	9.4–9.6	9.6–12.7	9.5
Ca^{2+}	10.65–11.6	9.5–9.9	28.2
Mg^{2+}	57–60	48.2–50.7	47.2
Cl^-	472–480	520.1–552.6	476
SO_4^{2-}	46.1–47.3	26.2–33.3	47.2
HCO_3^-	4.2–5.2 (CO_2)	2.4–5.2	7.6
pH	7.33–7.41	8.4–8.53	7.5

* Analysis 1 from Crenshaw, 1972b. Range of data from 3 species of marine molluscs.

** Analysis 2 from Wada and Fujinuki, 1976. Range of data from 4 species of marine molluscs.

*** AIEF, artificial inorganic extrapallial fluid used in these experiments.

21 C ± 1°C. In a variation of this experiment, 1.0 ml of the test substance in a solution of AIEF was added to the flask when the pH reached 8.15 to assess the effect on crystals which were already forming.

Crystallization of $CaCO_3$ was followed by measurements of pH change resulting from protons released in the reaction

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+.$$

This method is similar to that used by Pytkowicz (1965), Chave and Suess (1970), Wheeler *et al.* (1981), Sikes and Wheeler (1983), and Borman *et al.* (1982). During nucleation and crystallization, the pH decreased from pH 8.33 to approximately pH 7.5. In so doing, the pH was within the range reported for extrapallial fluids under normal conditions (Table I). The length of time prior to initiation of crystal nucleation and pH decrease is termed induction time (Garside, 1982). For comparative purposes we have chosen to represent this length of time as a function of both the induction period and the crystallization rate. An illustration of our method is shown in Figure 1. Mean induction times were obtained for each of the test substances and their standard deviations were calculated.

The course of crystallization was also followed in a few experiments by simultaneous measurements of optical changes (Termine and Eanes, 1974) and pH. Similar, but not identical, changes occurred in both.

Extrapallial fluid experiments

Specimens of the oyster *Crassostrea virginica* and the clam *Mercenaria mercenaria* were collected near the Duke University Marine Laboratory at Beaufort, North Carolina. Extrapallial fluid (EPF) was withdrawn immediately by prying open the valves and inserting a needle under the mantle edge into the central extrapallial space and collecting the fluid in a sterile syringe. Samples thought to be contaminated by sea water were not used. EPF from several animals was pooled, kept on ice, and the first samples were used on the day of collection. Remaining samples were maintained on ice in a refrigerator at 4°C. The measured characteristics of these samples remained stable over 7 days.

Various amounts of EPF were added to AIEF and induction times of crystallization were measured. To ascertain whether the observed inhibitory activity of EPF was due in part to the presence of negatively charged groups, the fluid was passed through

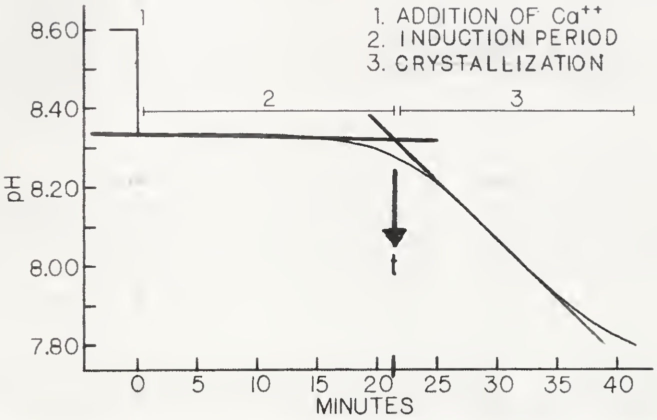

FIGURE 1. Chart recording of an actual control curve showing the 3 phases of *in vitro* $CaCO_3$ crystallization in artificial inorganic extrapallial fluid. Induction time (t) is indicated by the intersection of the 2 lines drawn along phases 2 and 3 on the graph.

a 9 × 20 mm DEAE-Sephadex column. Preliminary experiments were undertaken to determine the appropriate pH of the buffer and resin. A pH of 7.0 was selected and 2 columns were prepared with resin allowed to swell in AIEF, pH 7.0, for 2 days. The columns were then poured and washed with 50 volumes of AIEF and rewashed with 20 volumes 0.05 Tris buffer at the same pH. Measurement of the induction time of EPF, (200 μl EPF per ml of 0.05 *M* Tris buffer), was carried out. A 1.35 ml sample of this dilution was then applied to each of 2 columns and a 1.25 ml aliquot was collected from each. This material was assayed against a control column-run sample which contained buffer alone. The columns were then eluted with 1.35 ml of 0.5 *M* NaCl in Tris buffer, and a second elution was made using 2.0 *M* NaCl in 0.05 *M* Tris buffer. Samples of unbound and eluted materials were assayed in duplicate.

Magnesium Experiments

The effect of Mg on the induction time of crystallization was measured in magnesium-free AIEF to which $MgCl_2$ was added to give final concentrations of 0.5–120 m*M*.

Amino acid experiments

Seven free and 5 polyamino acids (Sigma Chemicals) were studied for their effects on $CaCO_3$ crystallization. The free amino acids included aspartic and glutamic acids, glycine, leucine, lysine, histidine, and taurine. Polyamino acids tested were polyaspartic and polyglutamic acids, polyglycine, polyleucine, polylysine, and polyhistidine. All free amino acids were tested at concentrations up to 1.47×10^{-4} *M*.

Because the polyamino acids differed greatly in their degree of polymerization, concentrations were standardized by preparing dilutions to contain equal numbers of amino acid residues. Concentrations are expressed in this way to allow ready comparison between the different polyamino acids regardless of their degree of polymerization. The polyamino acids were tested at as nearly equivalent concentrations as possible except in the case of polyaspartic and polyglutamic acids which inhibited crystallization so markedly that direct comparison with other polyamino acids was impractical. In these cases, lower concentrations were investigated to determine the lowest concentrations capable of producing significant inhibition of $CaCO_3$ crystallization.

RESULTS

All experimental and control runs produced sigmoid curves between the point marked by the addition of $CaCl_2$ (pH 8.33), and pH 7.80, the point at which $CaCO_3$ formation was essentially complete (Fig. 1). Artificial inorganic extrapallial fluid (AIEF) to which no $CaCl_2$ had been added gave a straight line trace on the chart recorder for a week or more with a pH decrease of only a few hundredths of a pH unit.

The induction times of crystallization were compared in samples of extrapallial fluid prior to and following passage through DEAE-Sephadex columns which bind compounds with negatively charged groups. Whole EPF was assayed at concentrations ranging from 0.1 μg/ml (7 μl) to 14.0 μg/ml (980 μl). At 3 μg/ml (210 μl EPF/ml) induction time increased to ≃800% of control time. At 14.0 μg/ml crystallization had not taken place after 2 days. The following experiment illustrates the results after the passage of EPF through DEAE columns. A sample containing 200 μl (2.9 μg/ml) of EPF in 0.05 *M* Tris buffer, pH 7.0, had an induction time of >3 h. The induction time was reduced to 18.5 min after this material was passed through a DEAE column. This material contained a Mg concentration roughly equivalent to the concentration found in the control material as the column does not effect the cationic components of material applied. Since the mean control time for the sample without EPF was 20.5 min, it appears that all inhibitory activity had been removed by the column. After elution of the column with 2.0 *M* NaCl, the induction time of the eluate was again >3 h, as compared to a 15 min control elution, demonstrating recovery of the bound inhibitory material from the column.

Induction time of crystallization increased with Mg concentrations of 5 m*M* Mg and above (Table II). At 47 m*M*, the equivalent of total Mg present in the extrapallial fluid of marine bivalves (Crenshaw, 1972b; Wada and Fujinuki, 1976), the induction time was several fold greater than that produced in the absence of Mg.

All free amino acids resulted in mean induction times which were not significantly different statistically from the control mean even at the highest concentrations tested (1.47×10^{-4} m*M*). Polyamino acid solutions containing the same number of residues per volume were compared. Polyaspartic and polyglutamic acids produced significantly greater induction times at concentrations equal to or lower than those of the corresponding free amino acids. Polyaspartic acid was inhibitory at concentrations of 7.36×10^{-8} *M* residues and above. The average length of induction time as a function of residue concentration for polyaspartic and polyglutamic acids is shown in Table III. These data imply an exponential increase in induction time with increasing polyamino acid concentration. Polyhistidine, polyglycine, and polylysine lacking carboxyl groups produced no significant increase in induction times even when tested at concentrations 100 times greater than those which produced a minimal effect with

TABLE II

Summary of data from in vitro $CaCO_3$ crystallization experiments

Test material	Concentration	Induction time in minutes (+S.D.)
control	0	21.5 (±7.4)
magnesium	0.5 m*M*	26.0 (±4.0)
	5.0 m*M*	48.5 (±1.8)
	29.2 m*M*	55.7 (±4.3)
	47.2 m*M*	190.0 (±2.0)
	117.0 m*M*	>23 h
whole EPF*	Tris buffer p̄ column control	20.5 (±2.0)
	200 μl/ml. buffer	>3 h
	EPF post column	18.5 (±3.5)
	EPF p̄ column elution (2.0 *M* NaCl)	>3 h
	Control elution (2.0 *M* NaCl)	15.0

* Extrapallial fluid obtained from *Mercenaria mercenaria.*

the negatively charged polyglutamic and polyaspartic acids. Our results with free amino and polyamino acids support those of Sikes and Wheeler (1983), carried out in artificial sea water adjusted to pH 9.5. These investigators observed that only negatively charged polyamino acids produced an inhibitory effect on mineralization.

In a variation of the experiments reported above, polyglutamic acid (pH adjusted to 8.15) when added to the flask at a concentration of 1.47×10^{-6} *M* residues *after* crystallization had begun, immediately arrested crystal formation and produced an induction time which was significantly longer than the control (Fig. 2).

DISCUSSION

Artificial inorganic extrapallial fluid (AIEF) to which sufficient Ca has been added, will form crystals of $CaCO_3$ after an induction period which depends upon conditions in the fluid (see Mullin, 1972). Using inorganic extrapallial fluid (AIEF) with added Ca, we have demonstrated that extrapallial fluid (EPF) in high dilution markedly decreases the rate of $CaCO_3$ crystallization. This inhibition was removed by passage

TABLE III

Mean induction times for various concentrations of polyaspartic and polyglutamic acids

Sample	Concentration*	Mean induction time in minutes (+S.D.)
Control (no amino acids)	0	23.5 (±8.3)
Polyaspartic acid	7.36×10^{-9} *M*	27.0 (±0.28)
	1.47×10^{-8} *M*	24.7 (±1.4)
	**7.36×10^{-8} *M*	52.3 (±4.0)
	1.47×10^{-7} *M*	115.7 (±25.9)
	7.36×10^{-7} *M*	48.0 h (±2.2h)
Polyglutamic acid	7.36×10^{-8} *M*	25.2 (± 4.5)
	**1.47×10^{-7} *M*	124.0 (±5.0)
	1.47×10^{-4} *M*	18.1 h

* Concentrations expressed in *M* residues.

** Concentration at which inhibition can be detected

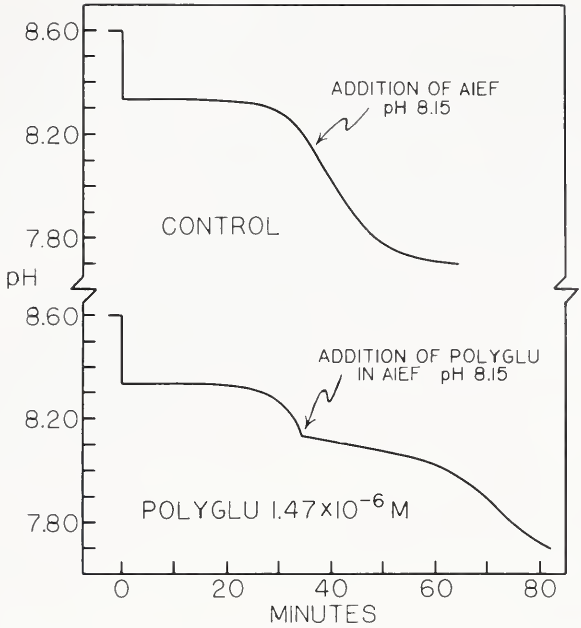

FIGURE 2. Time course of $CaCO_3$ crystallization in artificial inorganic extrapallial fluid (AIEF). Lower curve shows temporary retardation of crystal growth on addition of polyglutamic acid in AIEF, pH 8.15, after crystallization had begun. Upper curve demonstrates the absence of inhibition upon addition of AIEF, pH 8.15, without polyglutamic acid at a corresponding time.

through DEAE-Sephadex indicating that the inhibition was produced by negative groups. Carboxyl groups of proteins and carbohydrates could be involved since proteins (Pietrzak *et al.,* 1973) and carbohydrate (Misogianes and Chasteen, 1979) are present in extrapallial fluid. However, other anionic groups of polypeptides including phosphate (see Sikes and Wheeler, 1983) and anionic groups of polysaccharides and organic acids of metabolism cannot be excluded. Both COO^- and SO_4^{2-} have been considered possible inhibitors of crystal growth (Crenshaw and Ristedt, 1976; Weiner and Traub, 1981). Borman *et al.* (1982) found that the carboxyl groups, but not the ester sulfate groups, of the polysaccharide of coccoliths of *Emiliana* were important in inhibiting $CaCO_3$ crystallization *in vitro.*

Wheeler *et al.* (1981) and Sikes and Wheeler (1983) found that soluble shell matrix inhibited the formation of $CaCO_3$ crystals. This was presumed to be due to the negatively charged groups associated with protein in *Crassostrea.* Inhibition of crystal formation *in vitro* by polyaspartic and polyglutamic acids which have multiple COO^- groups (Sikes and Wheeler, 1983; this study) supports this explanation. The inhibition of crystallization by polyamino acids with acidic residues, but not by equivalent concentrations of the corresponding free amino acids, demonstrates that multiple COO^- groups in a polypeptide chain are required for inhibition (Sikes and Wheeler, 1983; Wilbur and Bernhardt 1982). Attachment of the chains to crystal nuclei and crystal surfaces probably interferes with growth of the lattice. Complete inhibition of crystal formation with sufficient concentrations of extrapallial fluid and acidic polyamino acids observed in the present experiments is presumably the result of the binding of acidic groups to crystal nuclei (Borman *et al.,* 1982). Calculations indicated that the maximum calcium reduction in our system through binding by free and polyamino acids did not reduce the free calcium content of the solution below supersaturation and did not exceed 0.4% change in calcium concentration.

Magnesium was found to decrease the rate of crystallization. In extrapallial fluid of marine bivalves the total Mg concentration is 49–60 m*M* (Crenshaw, 1972b; Wada and Fujinuki, 1976). The *in vitro* induction time for $CaCO_3$ in this range of concentrations was prolonged (Table II), and it follows that the Mg concentration in EPF may also retard crystal formation *in vivo.* Pytkowicz (1965) found that Mg also extended the induction time of the precipitation of $CaCO_3$ in sea water. In both sea water and EPF of marine bivalves, the concentration of Mg is some 5-fold that of Ca (Crenshaw, 1972b; Wada and Fujinuki, 1976). Pytkowicz (1965) points out that under such conditions collisions producing Mg-Ca-CO_3 aggregates will be more frequent than collisions resulting in $CaCO_3$. Accordingly, induction time will be increased.

The observed inhibitory effects of extrapallial fluid, negatively charged polyamino acids, and Mg on $CaCO_3$ crystallization *in vitro* raises the question as to the relationship between the experimental findings and $CaCO_3$ deposition by the organism. It will be evident that an extrapolation from the *in vitro* results to the mollusc is scarcely permissible in view of the differences between the two systems and the probable changes in the extrapallial fluid during the course of shell deposition. These differences notwithstanding, it may be that the inhibitory effects of organic compounds with negative groups and Mg could play a role in governing the rate of $CaCO_3$ deposition in molluscan shell.

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THE EYE STRUCTURE OF THE BIOLUMINESCENT FIREWORM OF BERMUDA, *ODONTOSYLLIS ENOPLA*

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ABSTRACT

The polychaete annelid, *Odontosyllis enopla* Verril of the family syllidae in the Bermudas, possesses a lunar periodicity and bioluminesces during the mating period. *Odontosyllis* has four eyes arranged so that two are located on each side of its head. The eyes are situated on lobes that have some degree of movement. Light sensitivity is maximum for illumination along the optical axis, which is different for the anterior and posterior pairs. The eye resides in a cavity formed by the pigment granules, and is structured of a lens, photoreceptor cells, the retina, and pigment granules. The retinal photoreceptors approach and surround the lens. The photoreceptors are formed of membranous processes, lamellae, and are similar in structure to the rhabdomeric photoreceptors of arthropods, cephalopod mollusc eyes, and the retinal rod outer segment of vertebrate eyes. The lens is a spheroidal body composed of cells. Closely associated with the lens are rods (tubes) about 60 nm in diameter arranged in linear arrays, suggesting that they are fiber optic bundles. The function of the fiber optic system could be to detect the direction of the bioluminescent light and to maximize the light-collecting ability of the eye.

INTRODUCTION

In the search for evolutionary clues in the development of the eye and vision, a comparative study of the eyes in a variety of invertebrate marine animals has been under continuous investigation (Wolken and Florida, 1969; Wolken, 1971, 1974, 1975). Among invertebrates, the arthropods and molluscs, evolved imaging eyes, and the question arose whether among annelids image forming eyes also evolved.

The eye structure of polychaete annelid was described some time ago by Greef (1877), Andrews (1892), and later by Hesse (1899). More recently Hermans and Eakin (1974) investigated by electron microscopy the fine structure of the eye on an alciopid, *Vanadis tangensis.* This was followed by Wald and Rayport (1977), who demonstrated that the alciopids *Torrea candida,* a surface worm, and *Vanadis,* found in the deep sea, possess image resolving eyes. To investigate the visual system of *Odontosyllis enopla* Verril, of the family syllidae, known as the "fireworm" of Bermuda, was of interest to us due to the early studies of Galloway and Welch (1911). *Odontosyllis* possesses a lunar periodicity throughout the year. The mating period continues from one to three days or more after the full moon, occuring precisely 50–55 minutes after sunset (Markert *et al.,* 1961). The females luminesce at the surface, attracting the males, who also luminesce (Huntsman, 1948). The bioluminescent color observed in its natural environment is bluish-green, but in the laboratory upon agitation, the

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live worms bioluminesce a bright green. The bioluminescent substance has been chemically identified as a luciferin system (Shimomura *et al.*, 1963; Trainor, 1979).

The response of *Odontosyllis* eyes to light stimuli of various wavelengths was determined by recording (ERG) electroretinogram (Wilkens and Wolken, 1981). The visual spectral peak response was around 510–520 nm, which coincides with the bioluminescent luciferin system emission peak of around 507–516 nm (Shimomura *et al.*, 1963; Nicol, 1978). This suggests that the response is a visual one for sensing the bioluminescent light. As a result of these studies we became interested in examining in greater detail how the eye of *Odontosyllis enopla* is structured for light reception.

MATERIALS AND METHODS

The Bermuda fireworms *Odontosyllis enopla* were collected during the months of July and August (1979–1981) in regions of shallow grass beds lining the shore of Whalebone Bay on the north shore of Bermuda. Both female and male worms were collected one hour after sunset and from one to five days after the full moon, by netting the worms at the surface during their bioluminescent mating displays.

For microscopy, both whole worms and the heads of worms were immediately fixed, some in 2.5% gluteraldehyde, pH 7.4, and others in Karnovsky's fixative, buffered with Millonig's phosphate buffer, pH 7.4. The fixed *Odontosyllis* were then refrigerated (4°C) until they were further processed for microscopic examination.

For electron microscopy, the fixed specimens were post fixed in 2% OsO_4 and the entire block stained with 2% aqueous uranyl acetate. All excess tissue was removed, and the organisms were dehydrated through a graded series of alcohols and finally in propylene oxide. They were placed in Mollenaur's number 2 formula for Epon-Aralidite and embedded in flat molds, in order to orient the eyes properly for sectioning.

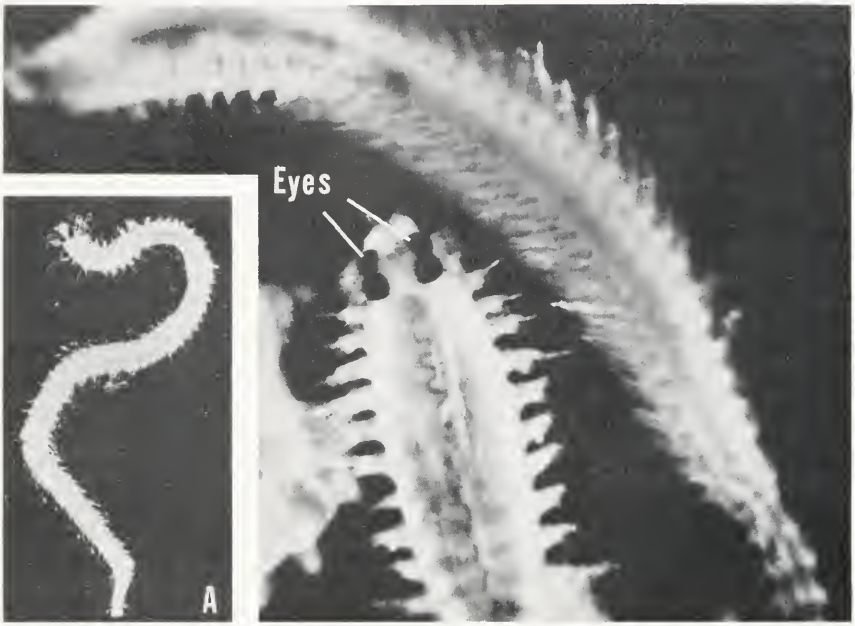
One micron sections were cut with glass knives on an LKB-Huxley Ultramicrotome. These sections were stained with methylene blue and examined by light microscopy. For transmission electron microscopy, the best fixed material was selected. Thin sections were then cut with a diamond knife on the Reichert Om-U2 Ultramicrotome. These sections were then double stained with 5% aqueous uranyl acetate for 20 minutes and then with Reynold's lead citrate for 5 minutes. The sections were scanned using the Philips Em-300 electron microscope, and various areas of the eye were photographed.

For scanning electron microscopy, the specimens were treated identically to those for transmission electron microscopy. The organisms were post fixed in OsO_4 and then dehydrated through a graded alcohol series to one hundred percent ethyl alcohol. The fixed and dehydrated organisms were dried in a Polaron Critical Point Dryer, mounted on studs, and coated in the Polaron E5100 sputter coater. The organisms were examined and photographed using the AMR-1000 scanning electron microscope in the Department of Biological Science, University of Pittsburgh.

To extract the visual pigment from the *Odontosyllis* eyes, 500 heads were dissected from the worms under red light in a cold room and were freeze dried. After thawing, the extraneous material was centrifuged away. The resulting material was then extracted with 1–2% aqueous digitonin in phosphate buffer, pH 7.5, in the dark at room temperature over night. The digitonin extract was then fractionated using a sucrose gradient density method followed by centrifugation and then ultracentrifugation (30,000 rpm for 1 hour). This resulted in a pink-colored layer that was removed from the centrifuge tube; its absorption spectrum was obtained in a Cary-14 Spectrophotometer.

RESULTS

Odontosyllis has four eyes arranged so that two pairs are located adjacent to each other on the dorsal surface of the head (Fig. 1). The eyes are on protruding lobes that have some movement (Fig. 2). There were individual differences in the locations



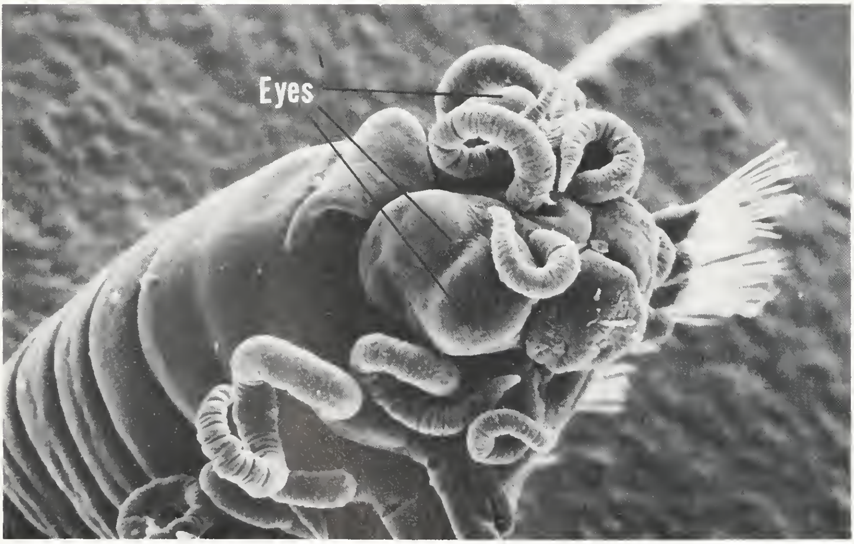
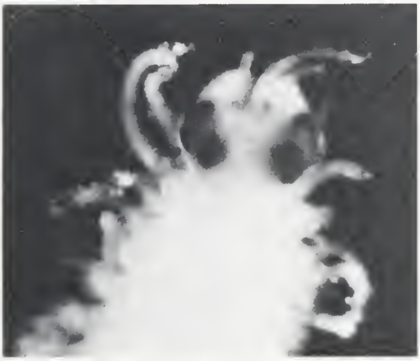


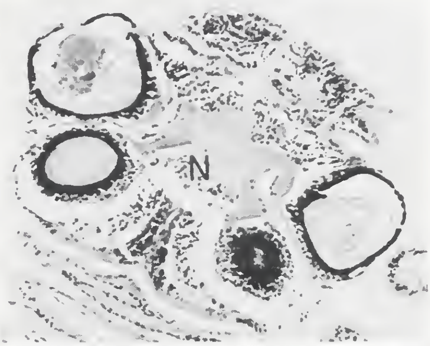
FIGURE 1. (Top) *Odontosyllis enopla* Verril. Live male and part of a female showing the two pairs of eyes. 13×. A. Live female (22 mm long, 1.5 mm in diameter). Photographed in laboratory with dark field illumination. 2.5×.

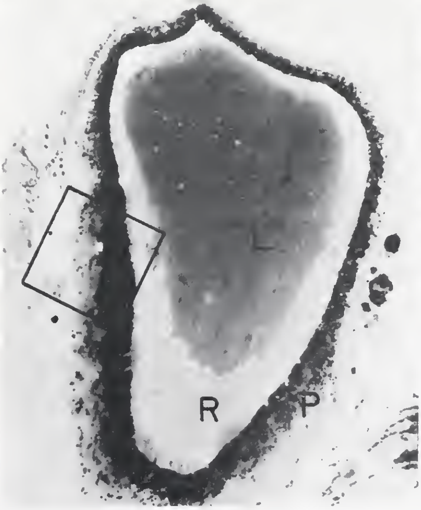
FIGURE 2. (Bottom) Scanning electron microscopy (SEM) of head area of *Odontosyllis*, showing lobes in which the two eyes are located. 106×.

of the eyes, as well as in their relative size. The eyes of the males are larger than the eyes of the females. The eyes seemed to change in size and pigmentation with time in the laboratory. In females collected during the breeding period, the eyes were heavily pigmented, with the anterior eyes being larger than the posterior ones (Figs. 3, 4). However, after 7–10 days in the laboratory, the anterior eyes had decreased in size and were more nearly the size of the posterior eyes, or smaller. In addition, both pairs of eyes had become less heavily pigmented, giving rise to the appearance of some of the inner structures of the eye.

The front of the eye is almost completely covered by the worm's cuticle, and each eye has a rounded exterior with a relatively small opening. The lens is located behind the opening. The position of the lens can only be distinguished from the rest of the darkly pigmented eye with the aid of a stereomicroscope. The lens is a spheroidal gelatinous body lying in the cavity formed by the pigmented cup (Fig. 5). From the







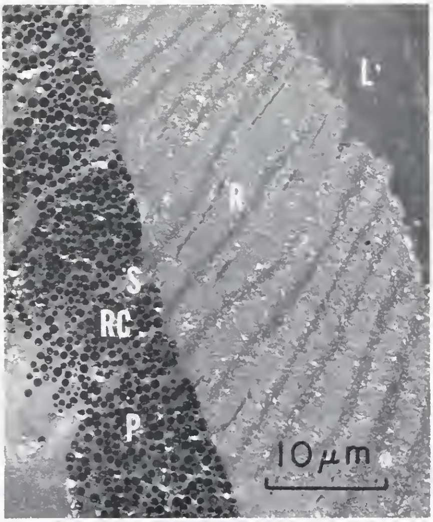


FIGURE 3. (Top left) Preserved female worm, with the two eyes on each side of the head anterior segments. 17×.

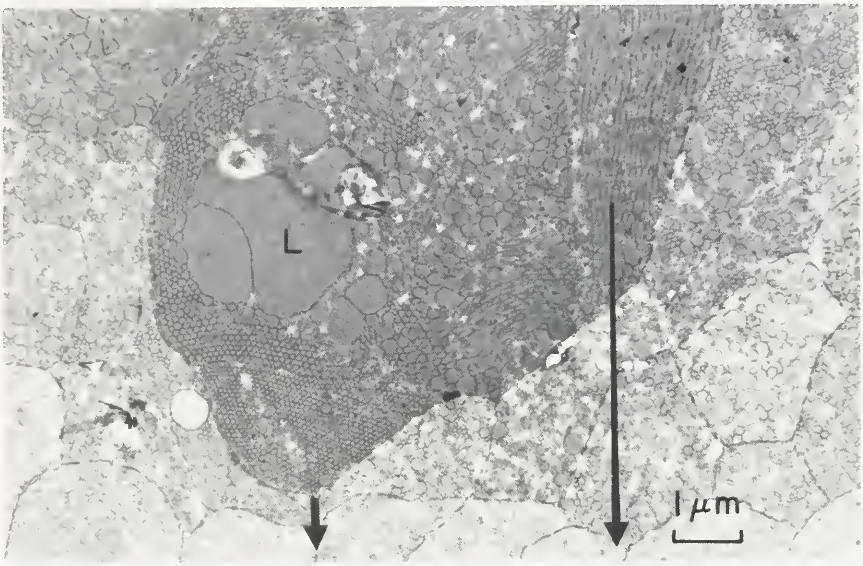
FIGURE 4. (Top right) Cross-section of a cut through the head, histologically stained, showing the two eyes on each side of the head and their relative size. N, region of neural structures. 92×.

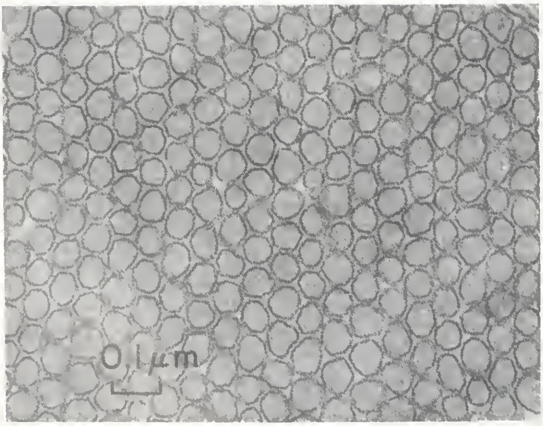
FIGURE 5. (Bottom left) Section through the eye, showing the lens (L), photoreceptors (R), and pigment granules (P) that surround the eye. EM. 306×.

FIGURE 6. (Bottom right) Higher magnification of boxed area in Figure 5, showing part of the lens (L), photoreceptors (R) and pigment granules (P), receptor cells (RC), supporting cells (S). EM.

contour of the eyes and the location of the lens, the optical axis of the anterior eyes appears to be directed forward and lateral whereas the axis of the posterior eyes appears to be directed dorsally, with a slight forward-lateral bias (Wilkens and Wolken, 1981). A cross-section cut through the eye shows the lens, photoreceptors, and pigment granules (Figs. 4–6). Microscopic examination of sections of the lens show that it is composed of cells. Electron microscopy of the lens indicated that within the lens area there are rods or tubes about 60 nm in diameter that are arranged in a uniform linear array (Figs. 7–9).

The retinal photoreceptors surround the lens and appear to be in contact with the lens (Figs. 5, 6). The retinal photoreceptors vary in length and measure up to 45





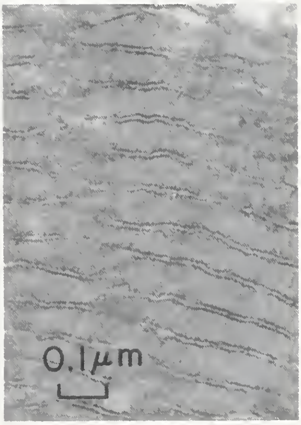


FIGURE 7. (Top) The lens, cells, and tubular structures in the lens. Compare shape of lens to Figure 5.

FIGURE 8. (Bottom left) The tubules, higher magnification of Figure 7, in cross-section arranged in linear arrays in the lens. EM.

FIGURE 9. (Bottom right) The lens, higher magnification of Figure 8, longitudinal section of the tubules.

μm and are about 1–2 μm in width (Fig. 10). The retinal photoreceptors are formed by membranous processes into lamellae that are about 70 nm in thickness (Fig. 10). The lamellae are aligned with the optical path and lie at right angles to the light path.

The retinal cells are separated by an extension of "grey" cell material and by mitochondria. The "grey" cells are located at the posterior end of the photoreceptors and are adjacent to them (Fig. 6). They contain the bulk of the pigment granules and form the inner part of the enclosure of the eye. There are openings (appearing circular) through or between these "grey" cells in which mitochondria are observed that are continuous with the regions that separate the photoreceptors. Myelinated bodies appear in these cells and in the cells adjoining them. There are also neural structures in the cells lying behind the photoreceptors which connect directly to the "grey" cells and to supporting cells that penetrate into this cellular region. Formalin fixed histological sections stained with hematoxylin and eosin indicated that neural fibers were located in the region (Fig. 4). The generalized eye structure of *Odontosyllis*, obtained from examining numerous microscopic sections (Figs. 3–10), was used to reconstruct the schematized eye in Figure 11.

It was also of interest to see if we could determine the nature of the visual pigment. Spectral analysis of the pink layer obtained from sucrose density fractionation showed two absorption peaks, one a sharp band in the neighborhood of 420–430 nm and another broader band around 500 nm. The spectral absorption peak around 500 nm could indicate that the visual pigment is a rhodopsin. However, our attempts to

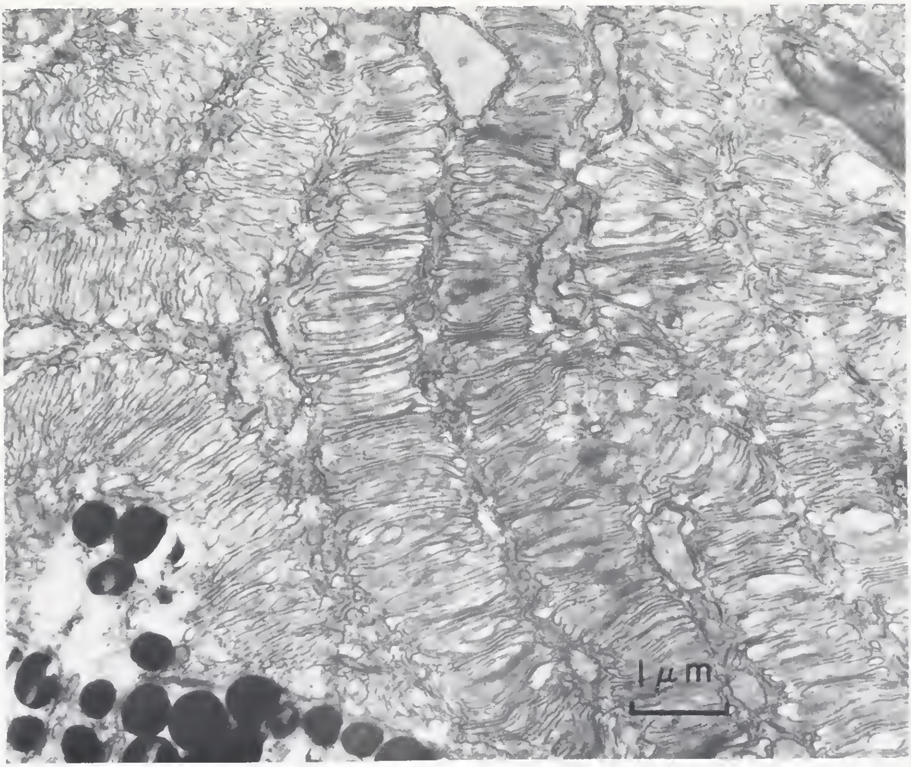


FIGURE 10. The retinal photoreceptors.

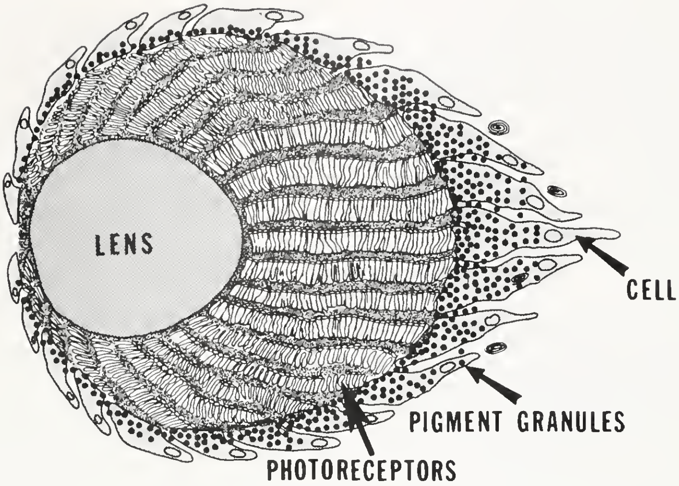


FIGURE 11. Schematic structure of the eye, showing lens photoreceptors, retinal cells, and pigment granules.

identify the visual pigment were limited due to the number of *Odontosyllis* heads (500) we could dissect and fractionate for analysis.

DISCUSSION

Considerable information has accumulated over the past century on annelid polychaetes, but their eye structure and visual physiology has not been extensively investigated, partly because it was believed until recently that they did not evolve image forming eyes and that many marine polychaetes of interest are rare and difficult to collect alive (Wald and Rayport, 1977).

As already noted, the electroretinogram (ERG) of the eyes of *Odontosyllis,* the visual spectrum, was found to coincide with the bioluminescent emission spectrum (Wilkens and Wolken, 1981). This indicated that the *Odontosyllis* eye detects the bioluminescent light signals. The questions we then raised were how is the eye structured for imaging and what is its visual pigment? We were also interested to learn how the eye structure differs from that of the alciopids that do possess image resolving eyes (Wald and Rayport, 1977).

In general, the structure of the eye of *Odontosyllis enopla* as we have described it, Figures 1–10, and schematized it in Figure 11, does not differ greatly from the description and early drawings of the eye by Galloway and Welch (1911). More recent studies of the eye by electron microscopy of a closely related species, *Odontosyllis ctenstoma,* found off the coast of Roscoff, France (Bocquet, 1977, 1983), indicate similarities to the mature *Odontosyllis enopla,* including the observation that the eye undergoes hypertrophy (Bocquet, 1977). The eye structure does not differ greatly from other polychaetes, for example *Platynereis dumerilii,* as studied by Fischer (1971). Of interest to us was that within the lens area there are rods (tubes) arranged in a linear array that appear to be aligned with the optical axis (Figs. 7–9). Such an arrangement for the lens could function as light guides to maximize the light collecting ability for the eye. If the rods behave in this fashion, the lens would possess a fiber-optics system serving as an efficient light collector for the photoreceptors. Such a system would be consistent with the *Odontosyllis* environment, for successful mating depends upon the detection and location of the bioluminescent light. However, further studies of the optics of the eye beyond that of a light detecting system is necessary to resolve the question of how good an imaging system it possesses.

The retinal photoreceptor structure appears to be of rhabdomeric origin (Westfall, 1982). They are similar to the photoreceptors of arthropods, mollusc eyes, and vertebrate retinal rod outer segments (Wolken, 1971, 1975). In the alciopid *Vanadis* there is a unique accessory retina which is not found in *Torrea,* a surface worm. The additional retina for *Vanadis* was postulated by Wald and Rayport (1977) to act in conjunction with the main retina as a depth indicator (Waterman, 1974). This function is based on the differing rates of extinction with the depth of the wavelengths that penetrate the sea. The possession of two retinas permits greater sensitivity to the changing wavelengths of light. The anatomical arrangement of an accessory retina in these alciopid eyes appears to correspond with the multi-retinas found in deep-sea cephalopod mollusc and fish eyes. No such mechanism occurs in the eyes of *Odontosyllis,* which is consistent with its shallow water habitat. As to the nature of the visual pigment of *Odontosyllis,* spectral absorption indicates that it is a carotenoid, a rhodopsin system. However, a larger sample and further purification is necessary to identify more precisely the visual pigment.

The structure of the *Odontosyllis* eye as we visualize it could indicate that among the annelid polychaetes an evolutionary development occurred from that of a pinhole-type eye to that of a simple camera image-forming eye.

ACKNOWLEDGMENTS

The authors would like to acknowledge the collaboration and assistance of Lon A. Wilkens in the early part of this study. This research was supported in part from the Pennsylvania Lions Eye Research Foundation and from the National Science Foundation to J. J. Wolken at the Bermuda Biological Station (BBS 967).

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SPECTRAL SENSITIVITY OF THE SPINY LOBSTER, *PANULIRUS ARGUS*

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ABSTRACT

The spectral sensitivity of receptor cells in the eye of the spiny lobster *Panulirus argus* was measured with intracellular electrodes. All cells sampled were maximally sensitive at about 510 nm. Several of these retinular cells were filled with Lucifer Yellow-CH from the recording electrode, and subsequent histological examination showed that they contributed to the main rhabdom and their axons terminated in the *lamina ganglionaris.* Microspectrophotometry of the main rhabdom revealed a single visual pigment with λ_{max} at about 510 nm. Using extracellular recording and selective adaptation with colored lights, however, a second receptor type was unmasked, with peak sensitivity at 370 nm in the UV. Indirect evidence suggests that it is the small, eighth retinular cell located distal to the main rhabdom, which has been shown in crayfish to contain a visual pigment maximally sensitive at short wavelengths and to make a comparably small contribution to the ERG.

INTRODUCTION

The structural organization of the compound eye of the lobster, *Panulirus,* has been studied by several researchers (Parker, 1891; Eguchi and Waterman, 1966, Meyer-Rochow 1975). The adult eye is of superposition (scotopic or clear zone) morphology, in which the crystalline cones are separated from the retinular cell layers by a wide transparent region. Seven retinular cells contribute alternating layers of microvilli to a central rhabdom, which has an unusual shape in which the lobed proximal region is more than 10 times the diameter of the thin distal neck. The eye also contains an eighth retinular cell which lies distal to the main rhabdom.

Physiological studies of the eye of *Panulirus argus* have been few, although the animal exhibits a repertoire of visually oriented behaviors. Waterman and Wiersma (1963) recorded the ERG, and Fernandez (1965) extracted a visual pigment with a λ_{max} at ~504 nm. The nature of the rhabdom of *Panulirus longipes* and its possible insensitivity to the plane of polarization was discussed by Meyer-Rochow (1975).

Panulirus argus is largely nocturnal, foraging in shallow water around tropical coral reefs (Kanciruk and Herrnkind, 1978). A notable exception to this general pattern occurs every fall when the entire population exhibits diurnal activity and engages in a highly organized migration of several km. Declining water temperatures in autumn trigger the migration, which lasts for several days. The animals form single file columns of up to 60 individuals and maintain the queues for the duration of their migration. The biological reason for this mass migration is unknown, as is the method of orientation while they travel.

MATERIALS AND METHODS

Animals

Specimens of the spiny lobster *Panulirus argus* were obtained from Carolina Biological Supply. They were kept for as long as two months under overhead fluorescent

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lights on a 12 h light:12 h dark cycle in aerated sea water aquaria and fed fresh mussels. The temperature of the room was maintained at 30°C.

Electrical recording

For electrophysiological recording, the animal was dark-adapted overnight, and the eyestalk was excised and all structures distal to the retinal layer were dissected free under dim red light. After being mounted in the recording chamber and submerged in saline, the preparation was dark-adapted for at least one hour. The eye was then pinned to a cork base in a lucite chamber filled with MBL crustacean saline (Pantin, 1934). Intracellular recording electrodes had 45–60 MΩ resistances when filled with 3 *M* KCl and immersed in marine crustacean saline. The reference electrode was a chlorided silver wire in a glass capillary tube which was filled with 2% agar in 3 *M* KCl. Micropipette electrodes were also used for measurement of the ERG by recording subcorneally in extracellular space. Electrical responses were viewed and photographed on the face of an oscilloscope.

Optical stimulator

Light (10 nm half band width) from a grating monochromator (Bausch and Lomb) supplied by a 500 W tungsten-iodide lamp was focused onto the eye using a combination of glass lenses. Intensity was controlled with a pair of counter-rotating quartz-inconel optical wedges. An electromagnetic shutter delivered flashes of light of 100 ms duration. The system was calibrated with a photodiode of known sensitivity (United Detector Technology).

In experiments involving selective adaptation, the adapting source was a microscope lamp fitted with appropriate filters. The light source was focused onto the eye from the same direction as the stimulus by using a beam splitter and lenses.

Spectral sensitivity

Spectral sensitivity was assessed by stimulating the eye with a series of monochromatic lights (10 nm half-band width) at 5–40 nm intervals between 350 nm and 600 nm. The 5 nm intervals were used around the sensitivity maximum, but not in all experiments. Stimuli were 100 ms, separated by dark periods of up to 1 minute. The spectrum was swept in both directions in order to compensate for any systematic drift in the sensitivity of the preparation. At each wavelength, several stimuli were presented and the wedges adjusted until the criterion response was elicited (using either intracellular responses or the ERG).

Selective adaptation

For selective adaptation, an appropriate filter was placed in the adapting light source: Corning CS3-71 (λ's > 475 nm) for the yellow light adaptation and Corning CS5-60 ($\lambda_{max} = 415$ nm, 90 nm band width at 50% maximal transmission) for blue light adaptation. Relative to the maximum value at about 440 nm, the quantum flux of the blue adapting light was about 0.24 at 380 nm and 0.06 at 500 nm. The wedge was opened to allow the full light intensity at 350 nm to be used as a stimulus. The adapting light was then turned on and the intensity increased until the responses at 350 nm were reduced to the criterion amplitude. The spectral sensitivity was then assessed by the method described in the previous paragraph.

Lucifer Yellow-CH fills

For marking individual cells, iontophoretic recording electrodes were filled at the tip with a 5% aqueous solution of Lucifer Yellow-CH (Sigma) and backfilled with 0.1 *M* LiCl. Cells were iontophoretically injected with dye by passing 100 ms, 10 nA hyperpolarizing pulses at a rate of 5 s^{-1} for 5 to 6 minutes.

Retinas were dissected from their eyestalks and fixed overnight in a solution of 4% formaldehyde (from paraformaldehyde) in a 0.1 *M* sodium phosphate buffer (pH 7.4) at 5°C, dehydrated through an alcohol series, embedded in paraplast (m.p. 56–58°C) and sectioned at 5 μm. The serial sections were examined under a Zeiss fluorescence microscope using a mercury lamp and a 430 nm interference filter in the excitation path. The image was passed through 460 and 530 nm long pass barrier filters and photographed.

Microspectrophotometry

Rhabdoms from dark-adapted lobsters were isolated by dissecting the retinal layer under dim red light. The tissue was crushed with a stout glass rod in 1 ml of MBL crustacean saline (Pantin, 1934) at 0°C. No fixative was present. Drops of the suspension were placed in a ring of silicone grease and sealed between coverslips for study by microspectrophotometry as described below, and measurements were made at room temperature (22° ± 2°C). Solutions were buffered with 0.04 *M* HEPES (N-2-hydroxyethylpiperaxine-N′-2-ethane sulfonic acid), but in some experiments, small droplets of 0.01 *N* NaOH were added to move the pH to more extreme basic values.

A laterally incident microbeam that was rectangular and measured 7 × 2 μm in the plane of the specimen was used for spectral recording. This microbeam was produced by a dual beam microspectrophotometer with a reference beam external to the microscope. The light source was a 6 V, 100 W tungsten lamp, used in conjunction with a Bausch and Lomb 250 mm focal length monochromator with the grating ruled at 600 lines per mm. For the measuring beam, an image of an aperture was projected onto the specimen with a Zeiss X32 Ultrafluar glycerin immersion objective mounted on the condenser carriage of the microscope. An identical lens was employed in the collecting system. The detector was an EMI 9558 QA photomultiplier tube. Further details can be found in Goldsmith (1978).

The scans were under computer program control and spectra were digitized at 1 nm intervals. The duration of a single scan was ~30 seconds. No photoconversion caused by the measuring beam was observed when the organelle was scanned a second time. Each interval reading was an average of 25 interrogations of the A/D converter.

RESULTS

Intracellular potentials were recorded from approximately five hundred retinular cells in the isolated eye cup preparation of the lobster. Figure 1 shows average spectral sensitivity curves derived from intracellular recordings of cells from dark-adapted animals, based on depolarizing criteria of 5 mV (n = 25) and 2 mV (n = 6). Both curves have their maxima 510 ± 5 nm. Yellow and blue light adaptation of the eye failed to alter the shape of the spectral sensitivity curve derived from intracellular responses, providing no evidence for electrical coupling between receptors of different spectral type (Fig. 2).

Lucifer fills of the 510 nm receptor revealed that it is associated with the main rhabdom and synapses in the *lamina ganglionaris.*

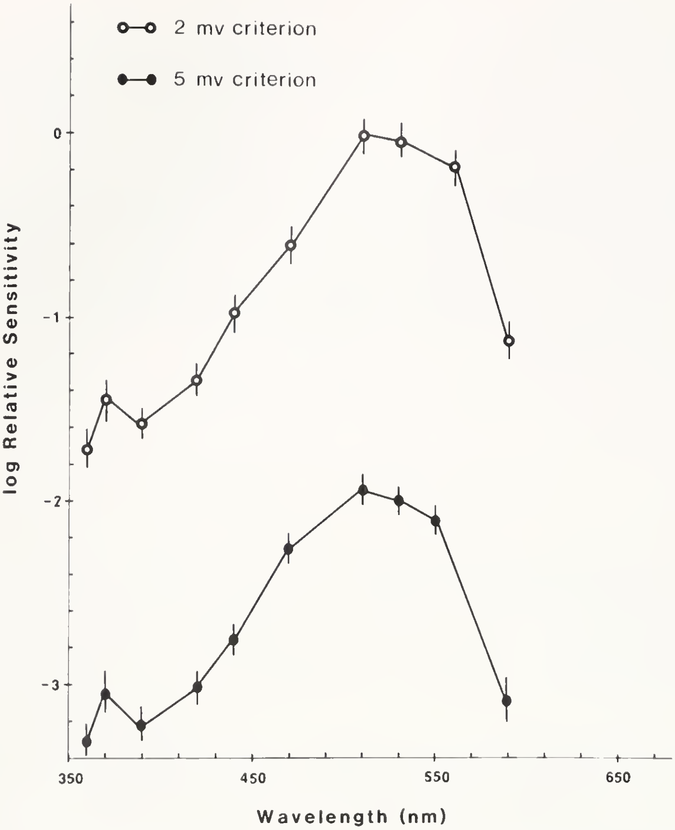

FIGURE 1. Spectral sensitivity curves of photoreceptor cells of *Panulirus argus* based on intracellular recordings. The upper curve is an average of 6 cells and is log reciprocal photon flux (in relative units) for a criterion response of 2 mV; the lower curve is an average of 25 cells and is based on a criterion depolarization of 5 mV. In both cases the peak sensitivity is at about 510 nm. Error bars indicate ±s.e.m.

As only a single spectral class of receptor was found in intracellular recording, experiments using the ERG were employed in order to unmask any possible contributions of other receptor types. The spectral sensitivity of the ERG of the dark-adapted eye was similar to the spectral sensitivity functions of individual cells, and the response-energy functions were parallel to each other at different wavelengths (not shown). But when the eye was adapted with colored lights and a very small criterion response employed, evidence appeared for a second type of receptor. The upper curve in Figure 3 is a spectral sensitivity function based on a 25 μV criterion response of the ERG from a dark adapted eye. When the eye was adapted with blue or orange background lights, however, the sensitivity not only decreased, but the shape of the spectral sensitivity function changed as well. Adaptation to long wavelengths accentuated the secondary maximum at 370 nm, whereas adaptation to short wavelengths abolished it. This effect was observed only with very small criterion responses (~25 μV). These experiments show the presence of two types of receptors, one having a maximum sensitivity in the green (λ_{max} 510 nm) and the other type

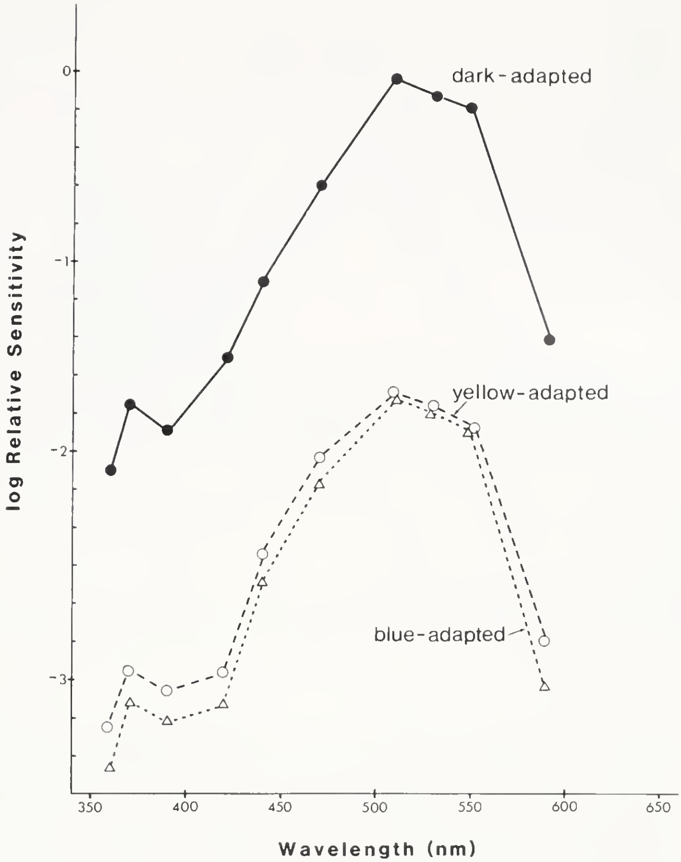

FIGURE 2. Spectral sensitivity of a dark adapted eye, derived from intracellular recording (filled circles, upper curve). The maximum sensitivity is at about 510 nm. When spectral sensitivity is measured in the presence of colored backgrounds, the shape of the spectral sensitivity function is unaltered (lower 2 curves).

having a maximum sensitivity in the ultraviolet (λ_{max} near 370 nm). The former seems the more abundant, as it dominates the sensitivity of the dark-adapted eye.

Microspectrophotometry of the main rhabdom demonstrated the presence of one pigment with a maximal absorption at 510 nm. Over 30 scans of randomly selected areas of main rhabdoms revealed only the 510 nm pigment. An example is shown in Figure 4. In order to obtain a difference spectrum, the pigment was bleached by orange light at pH 9, a treatment known to cause the photobleaching of crustacean rhodopsin (Bruno *et al.,* 1977). The metarhodopsin was not studied.

DISCUSSION

Panulirus rhodopsin in digitonin solution has λ_{max} at 504 nm (Fernandez, 1965), which is in reasonable agreement with both our electrophysiological and microspectrophotometric measurements. The data do not allow us to conclude that the 6 nm difference is real. Fernandez found the λ_{max} of *Panulirus* metarhodopsin at 495 nm.

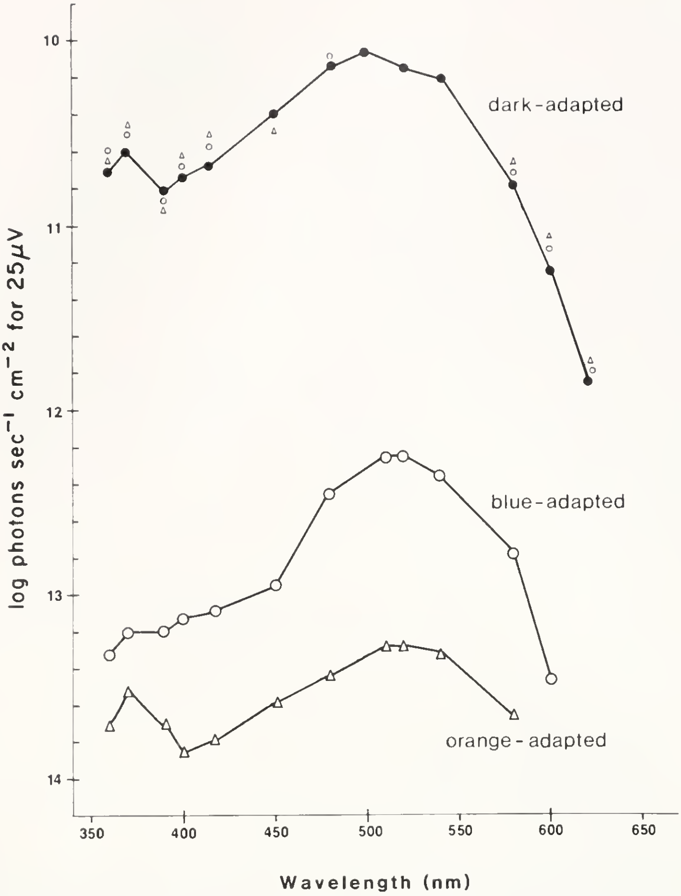

FIGURE 3. Spectral sensitivity of the dark-adapted eye, derived from extracellular recordings (filled circles and upper curve). The maximum sensitivity is at about 500 nm. When spectral sensitivity is measured in the presence of colored backgrounds, however, the shape of the spectral sensitivity function changes. This effect is observed only with small criterion responses, in this case 25 μV. The middle curve and large open circles show the suppression of the secondary maximum at 370 nm brought about by adaptation to blue light. Conversely, the lower curve and large open triangles show the enhancement of the 370 nm peak produced with an orange background. Small open circles and triangles show the recovery of sensitivity following the two adaptations.

The crayfish *Orconectes* and *Procambarus* (Goldsmith and Fernandez, 1968; Wald, 1968; Nosaki, 1969; and Waterman and Fernandez, 1970) and the prawn *Palaemonetes* (Goldsmith and Fernandez, 1968; Wald and Seldin, 1968) have two spectral types of receptor in the eye. In the crayfish the sensitivity maxima are at 560 and 440 nm; in the prawn, at about 550 and 380 nm. In each species the short wavelength receptors contribute little to the ERG, and in the case of the crayfish, intracellular recording and dye marking, combined with microspectrophotometric measurements, have shown the 440 nm receptor to be the small, distal eighth cell (Cummins and Goldsmith, 1981).

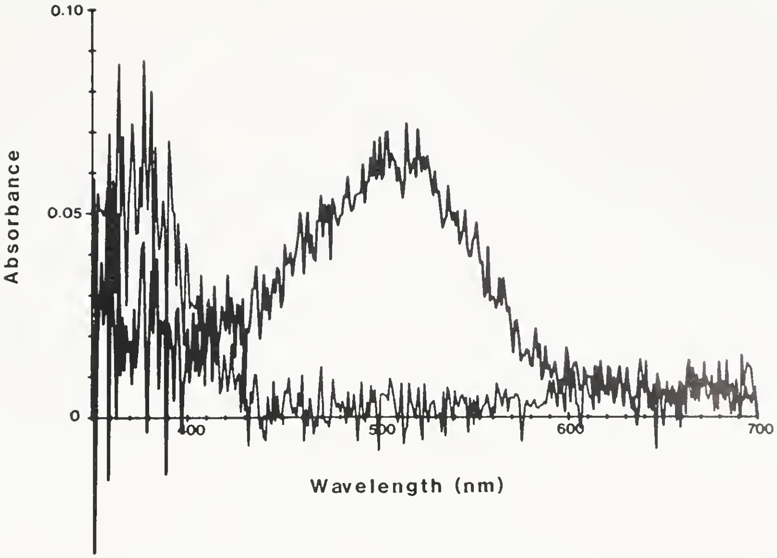

FIGURE 4. Microspectrophotometry of the main rhabdom reveals a visual pigment with peak absorption at about 510 nm (pH 7.5). At pH 9, several minutes exposure to a bright orange light causes the pigment to bleach to a photoproduct with peak absorption at about 370 nm, presumably retinaldehyde (left peak).

Extracellular recordings and selective adaptation experiments of the eye of *Panulirus* clearly indicate the presence of two spectral types of receptor in this species as well. The major contribution to the ERG comes from a receptor with a λ_{max} at 510 nm; a minor contribution is made by a receptor with a λ_{max} in the near UV. Furthermore, over 30 scans of random areas in the main rhabdom of the spiny lobster eye have shown the presence of only the 510 nm pigment. Although the 370 nm receptor was not picked up in the several hundred impalements made in all regions of the eye, we believe that, as in the crayfish, the short wavelength receptor is likely to be the eighth cell that is located distal to the main rhabdom. The fact that we failed to uncover the ultraviolet cell by intracellular recording could be due to several contributing factors, such as its small size, (5% of the rhabdom microvilli: Meyer-Rochow, 1975), its protection by its contiguous lenslet, or damage caused by removal of the more peripheral structures during preparation of the eye for recording.

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PATTERN FORMATION BY OOPLASMIC SEGREGATION IN ASCIDIAN EGGS*

WILLIAM R. JEFFERY

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ABSTRACT

This article reviews the generation of cytoplasmic patterns by ooplasmic segregation in ascidian eggs. Ascidian eggs exhibit a spectacular episode of ooplasmic segregation after fertilization which finalizes the position of many different constituents with respect to the future embryonic axes. Among the elements that are rearranged by ooplasmic segregation include three different cytoplasmic regions of unique morphogenetic fate, cytoplasmic organelles, cell surface components, the cytoskeleton, and localized maternal mRNA molecules. New experimental evidence is reviewed suggesting that the reorganization of these constituents into a specific pattern in the egg is coordinated by their association in a cytoplasmic complex. The contraction of an actin filament network in this complex after fertilization is proposed to be the motive force for ooplasmic segregation. The focal point for ooplasmic segregation may be controlled by a local increase in intracellular calcium at or near the point of sperm entry.

INTRODUCTION

All of the different cell types of adult metazoans are derived during embryogenesis from a single totipotent cell—the egg. One of the most challenging problems of embryology is how this array of different cell types is generated from the egg during early development. The first differences are detectible at very early stages in the development of determinative embryos. These differences are thought to be mediated by morphogenetic determinants localized in the egg cytoplasm and unequally partitioned to the embryonic cell lineages where they affect gene expression (see Wilson, 1925; Davidson, 1976; Jeffery and Raff, 1983 for reviews). The determinants may be formed during oogenesis, but their final arrangement is often dictated by an extensive series of cytoplasmic reorganizations known as ooplasmic segregation, which occur after fertilization or during the early cleavage cycles. The role of ooplasmic segregation in organizing the distributional patterns of morphogenetic determinants makes a thorough knowledge of this event of importance to understanding how cell fates are established during early development.

After being neglected for a number of years, interest in the process of ooplasmic segregation has now been rekindled among developmental biologists and some significant discoveries have recently been made. The purpose of this article is to review ooplasmic segregation in ascidian eggs emphasizing this newly-available information. First, we will present a short introduction to ooplasmic segregation and its role in the early development of ascidian embryos. Second, we will describe the events of ooplasmic segregation in detail emphasizing the concerted involvement of cytoplasmic

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* Editor's note: This article is based on a Friday Night Lecture delivered by Dr. Jeffery at the Marine Biological Laboratory on 19 August 1983.

organelles, the cell surface, the cytoskeleton, and localized maternal messenger RNA (mRNA) molecules. Finally, we will consider the mechanism of ooplasmic segregation and present models that have been developed to explain how the various constituents of the egg are placed in a developmentally-significant pattern prior to the first cleavage.

OOPLASMIC SEGREGATION AND ITS DEVELOPMENTAL SIGNIFICANCE

Ascidian eggs exhibit the most spectacular example of ooplasmic segregation that has yet to be described in nature. Unlike many other kinds of eggs, the visualization of ooplasmic segregation in certain ascidian eggs is greatly fascilitated by the presence of brilliantly-pigmented cytoplasmic regions. These pigmentations, which are due to the localization of specific cytoplasmic inclusions, and the fates of the regions of the egg they delineate were first noted by Conklin (1905) in *Styela partita* and have subsequently been reported in a number of different species of *Styela, Boltenia,* and *Pyura* (Table I). Other ascidian species have similar cytoplasmic inclusions in their eggs (*i.e., Ciona intestinalis;* Berg and Humphreys, 1960), but they are opaque or colorless and difficult to discern in the living egg (Reverberi, 1971).

Conklin (1905) reported five distinctly colored regions in the cytoplasm of *Styela partita* eggs, but only three of these are routinely distinguishable and can be followed with certainty during ooplasmic segregation. The following is a description of the ooplasmic organization of the unfertilized *Styela* egg. The myoplasm, which consists of yellow pigment granules and adhering mitochondria (Berg and Humphreys, 1960), encircles the entire periphery of the egg. The ectoplasm, a transparent region containing fine granular and membranous materials, which is originally derived from the sap of the germinal vesicle (GV), is present in the animal hemisphere of the egg. The endoplasm, colored gray and containing densely-packed yolk platelets, fills the remainder of the egg. Unfertilized *Boltenia* eggs consist of three cytoplasmic regions of similar composition, localization, and developmental fate as those of *Styela,* except that the myoplasm contains orange pigment granules (Berrill, 1948).

Ascidian eggs are shed as primary oocytes arrested in prophase of the first maturation division. Ooplasmic segregation and the completion of maturation is triggered

TABLE I

The colors of myoplasm and endoplasm in various species Styela, Boltenia, *and* Pyura

Species	Myoplasm	Endoplasm	Reference
S. partita	Yellow	Gray	Conklin (1905)
S. plicata	Yellow	White	Whittaker (1980)
S. clava	Yellow	Pink to Maroon	Jeffery (unpub.)
S. montereyensis	Light Green or Light Yellow	Dark Green	Miller cited in Abbott and Newberry (1980); Jeffery (unpub.)
B. echinata	Orange		Berrill (1948)
B. hirsuta	Orange	Colorless	Reverberi (1971)
B. villosa	Orange to Red	White	Jeffery (1982a); Abbott and Newberry (1980); Cloney (1961)
P. microcosmus	Brown	Pink-gray	Millar (1954)
P. squamulosa	Orange	Pink	Millar (1951)

by fertilization. The cytoplasmic rearrangements occur during a thirty minute period which encompasses maturation and part of the time between the completion of the second maturation division and the first cleavage. Two phases are apparent in the segregation process (Reverberi, 1961). The first phase occurs during the maturation divisions. It is probably initiated very shortly after sperm entry, when the myoplasm begins to stream down the periphery of the egg in a vegetal direction. According to Conklin (1905) the myoplasm streams toward the site of sperm entry, which is thought to be at or near the vegetal pole of the egg. The detailed features of the myoplasmic movements are ordinarily difficult to discern, even in intensely-pigmented eggs, mainly because of the opacity of the underlying yolk platelets. Extraction of segregating eggs with a non-ionic detergent (such as Triton X-100), however, dissolves the yolk platelets and has been found to highlight contrast between the myoplasmic pigment granules and adjacent areas of the egg (Fig. 1). By applying the detergent extraction procedure at various intervals during ooplasmic segregation, it has been shown that the downward movement of myoplasm is not uniform throughout the egg periphery. It appears to begin in the animal hemisphere and to progressively move toward the vegetal pole, collecting the pigment granules into a dense band at its upper margin as it proceeds (Figs. 1, 2) (Jeffery and Meier, 1983; Jeffery, unpub.). Sawada (1983) has recently

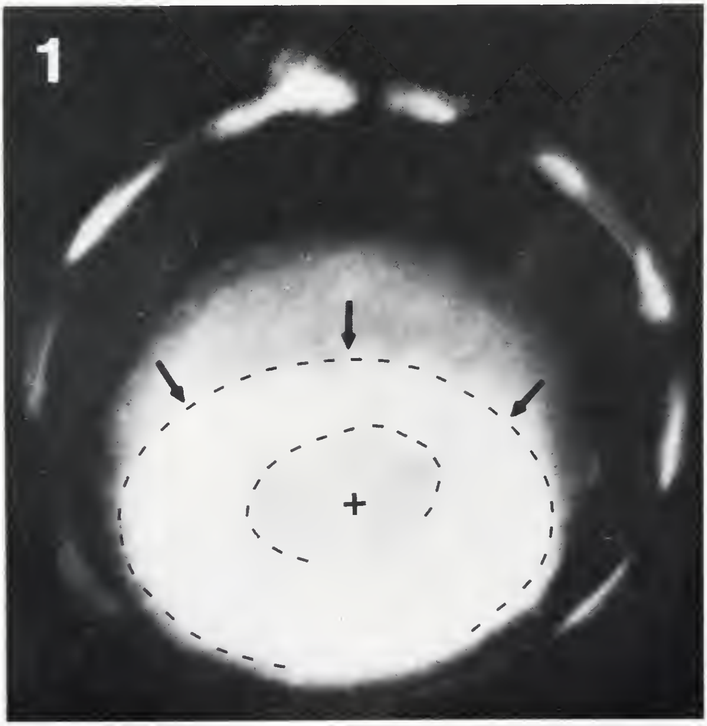

FIGURE 1. A dark field light micrograph of a *Styela plicata* egg viewed from the vegetal pole during the first phase of ooplasmic segregation. The egg was extracted with Triton X-100 as described by Jeffery and Meier (1983) to highlight the myoplasm, which can be seen as a bright ring of pigment granules in the process of streaming toward the vegetal pole. The ring of myoplasmic pigment granules is partially encircled by dotted lines. The approximate location of the vegetal pole is marked +. The arrows indicate the direction of myoplasmic streaming. The thin, bright halo around the egg is the remnant of the chorion. ×350.

shown that a thin peripheral layer of yolk platelets also streams vegetally along with the myoplasm in *C. intestinalis* eggs. As described below, however, most of the yolk moves in an animal direction during the first phase of segregation.

The marginal band of pigment granules does not reach the vegetal pole by the time the first phase of segregation is completed, and as a result the myoplasm in the polar region contains considerably fewer pigment granules and is tinted a lighter yellow than the immediately surrounding area (Fig. 2). The difference in myoplasmic coloration generated during ooplasmic segregation is maintained during later development and signifies the divergence of the larval tail muscle and mesenchyme cell lineages. The light yellow area (sometimes called the mesoplasm) eventually enters the mesenchyme cells and the surrounding darker yellow area enters the tail muscle cells (Conklin, 1905).

As the myoplasm flows into the vegetal hemisphere, most of the ectoplasm is divided into many separate islets of protoplasm in the animal hemisphere. These islets stream into the vegetal region of the egg in the wake of the myoplasm, temporarily come to rest, and then fuse again forming a transparent ectoplasmic band immediately

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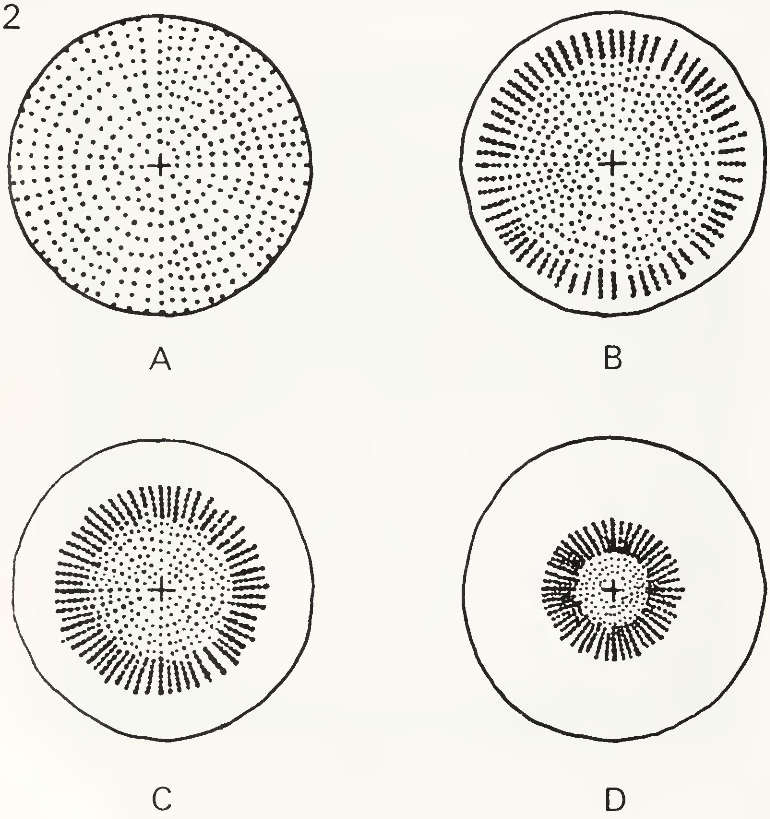


FIGURE 2. A diagrammatic representation of the movement of the myoplasmic pigment granules toward the vegetal pole of the *Styela* egg during the first phase of ooplasmic segregation. The egg surface is viewed from the vegetal pole (+). The filled circles represent individual myoplasmic pigment granules. The unfertilized condition is represented by A, while B–D represent progressive intervals during ooplasmic segregation.

above the vegetal cap of myoplasm. Most of the endoplasmic yolk platelets are displaced upwards into the animal hemisphere at the same time as the myoplasm and ectoplasm are streaming into the vegetal hemisphere. A wide protoplasmic lobe initially appears at the animal pole concomitant with the formation of the first polar body, and then at the vegetal pole at the time of the extrusion of the second polar body (Fig. 3) (Conklin, 1905; Zalokar, 1974; Sawada and Osanai, 1981). At the conclusion of the first stage of ooplasmic segregation the endoplasm, ectoplasm, and myoplasm are stratified perpendicular to the animal-vegetal axis of the egg.

The second phase of ooplasmic segregation begins at the completion of maturation. The male pronucleus forms an aster and begins to move toward the animal hemisphere, where it will eventually fuse with the female pronucleus. At this time the myoplasmic

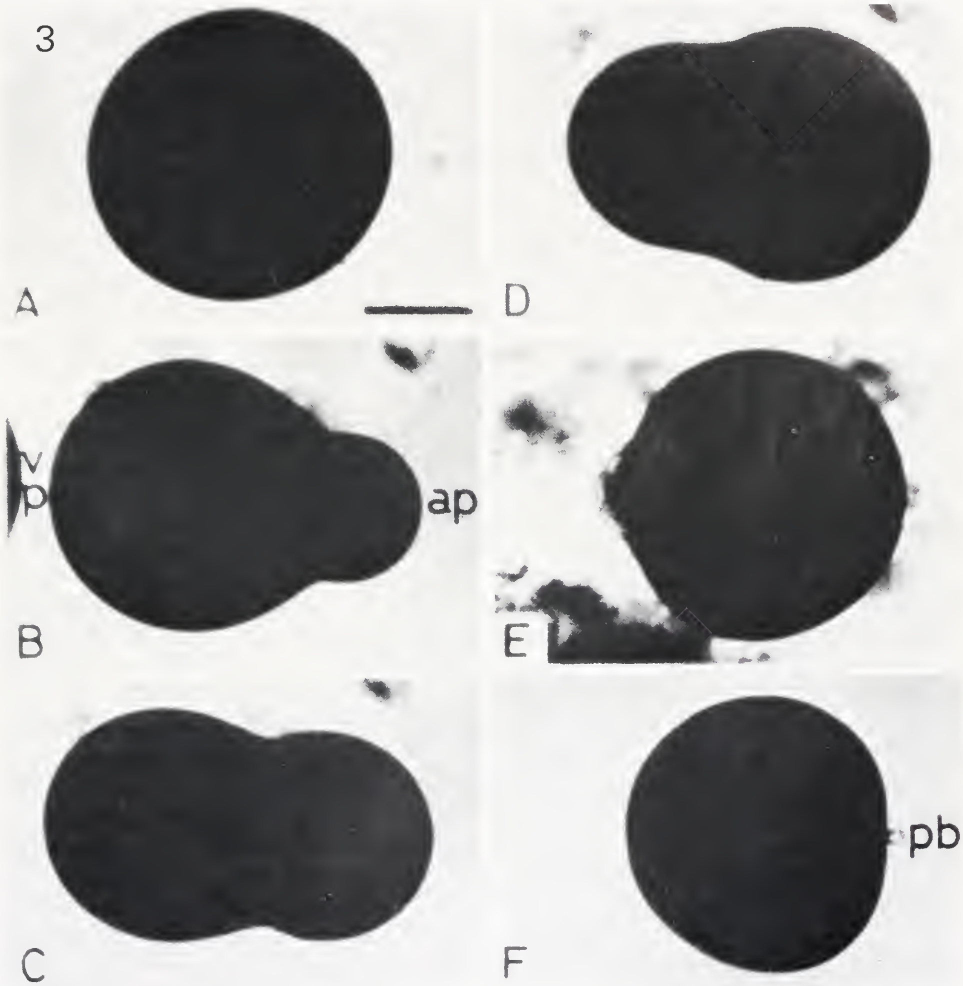


FIGURE 3. Light micrographs showing the modification of egg shape during ooplasmic segregation of dechorionated *Ciona intestinalis* eggs. A. An unfertilized egg. B–F. Successive shape changes that occur during the first phase of ooplasmic segregation. ap: Animal pole. vp: Vegetal pole. pb: Polar body. The bar in A represents 50 microns. From Sawada and Osanai (1981).

pigment granules, with their crown of ectoplasm, move upward along the vegetal periphery of the egg until they reach what will be the future posterior region of the embryo. At this location they spread out to form the myoplasmic crescent (the yellow crescent in *Styela;* Conklin, 1905). Very soon after the myoplasmic crescent is formed, the ectoplasm flows back into the animal hemisphere with the male pronucleus, where it forms a specialized cytoplasmic region around the synkaryon. At the same time, a large proportion of the endoplasmic yolk platelets return to the vegetal hemisphere. At the termination of ooplasmic segregation the ectoplasm is again located in the animal hemisphere surrounded by a thick layer of endoplasmic yolk platelets. The vegetal hemisphere, which is almost entirely filled with yolk platelets, contains the myoplasmic crescent at its posterior extremity. According to Conklin (1905), the region of the vegetal endoplasm opposite the myoplasmic crescent, which will become the anterior portion of the embryo, contains crescents of chordamesoplasm and neuroplasm.

By the conclusion of ooplasmic segregation the three major axes of the embryo are established, and ooplasmic patterns are formed in the egg which will be passed to the different cell lineages of the embryo during cleavage. The myoplasm is primarily distributed to the larval tail muscle and mesenchyme cells during embryogenesis, while the ectoplasm and endoplasm are primarily segregated into the ectodermal and endodermal cells of the embryo respectively (see Whittaker, 1979 and Nishida and Satoh, 1983 for reviews of ascidian cell lineages).

The dramatic cytoplasmic rearrangements that occur in ascidian eggs have at least two important morphogenetic consequences. First, these events appear to be responsible for setting up the final pattern of determinant distribution. Evidence for this role comes from fragmentation experiments performed on *Ciona intestinalis* and *Ascidia malaca* eggs by Reverberi (1931, 1937) and Reverberi and Ortolani (1962). They found that halves, cut in any plane of section from an unfertilized egg, each gave rise to miniature larva after fertilization. When the same cuts were made with fertilized eggs that had completed ooplasmic segregation, only the vegetal halves formed larvae. The animal fragments arrested as blastulae. Second, ooplasmic segregation seems to be responsible for determining bilateral symmetry in the embryo. As mentioned above, if unfertilized eggs are cut along the animal-vegetal axis, both halves are capable of forming complete larva. If this operation is done after the completion of ooplasmic segregation, however, complementary half-larvae are obtained from each egg half (Ortolani, 1958). These and other experiments (see Reverberi, 1961, 1971; Whittaker, 1979 for reviews) indicate that ascidian eggs are exceptionally determinative and that this property originates between the time of fertilization and the completion of ooplasmic segregation. The classical explanation of these results is that the process of ooplasmic segregation fixes the pattern of morphogenetic determinants in ways that are essential for the determination of the embryonic cell lineages. Recently, Whittaker (1980, 1982) has shown that the myoplasmic cells inherit transferable determinants that can promote the synthesis of acetylcholinesterase, an enzyme normally restricted to the tail muscle cells, in blastomeres that form non-muscle lineages.

OOPLASMIC SEGREGATION OF EGG SURFACE COMPONENTS

Since the first description of ooplasmic segregation in *Styela partita* eggs, many investigators (including Conklin, 1905) have noted that some of the test cells segregate with the myoplasm into the vegetal region of the egg forming a cellular layer above the myoplasmic crescent. Ascidian eggs are surrounded by a highly complex follicle which persists until the time of larval hatching. The follicle consists of three components: the follicle cells, which are exposed to the outside environment, the chorion, and the test cells, which lie between the chorion and the surface of the egg (Tucker, 1942; Kessel, 1962; Jeffery, 1980). The behavior of the test cells during ooplasmic segregation implies that they may be connected to the egg surface and that components of this surface also move into the vegetal region of the egg with the myoplasm during ooplasmic segregation. There may be a practical reason for the behavior of test cells after fertilization. The test cells are known to elaborate the tail fin and associated ornamentation in the larva of some species of ascidians (Cloney and Cavey, 1982). Co-segregation with the cytoplasmic region that eventually enters the tail muscle lineage may help place the test cells in the appropriate position to perform their subsequent embryonic function. The connections between some of the test cells and elements on the egg surface may be elaborated during late oogenesis when these cells are endocytotically embedded in the cytoplasm of the developing oocyte (Tucker, 1942; Kessel, 1962). Selective staining of the test cells in *Halocynthia roretzi* has revealed long, fine filaments that extend from the test cells to the surface of the oocyte (Hirai, 1949).

Several other kinds of observations suggest that cell surface components also participate in ooplasmic segregation in concert with the myoplasm. First, supernumerary sperm, chalk granules, or carmine particles bound to the surface of dechorionated *Ciona intestinalis* eggs gradually become concentrated in the vegetal hemisphere during ooplasmic segregation (Ortolani, 1955; Sawada and Osanai, 1981). Second, stout microvilli covering the surface of unfertilized *C. intestinalis* eggs become restricted to the egg surface over the myoplasm at the completion of ooplasmic segregation (Fig. 4) (Sawada and Osanai, 1981). Finally, lectin binding sites, which are present throughout the surface of the unfertilized eggs, also co-segregate with the myoplasm after fertilization (Monroy *et al.*, 1973; O'Dell *et al.*, 1974; Ortolani *et al.*, 1977; Zalokar, 1980). It seems likely that the reorganization of the cell surface during

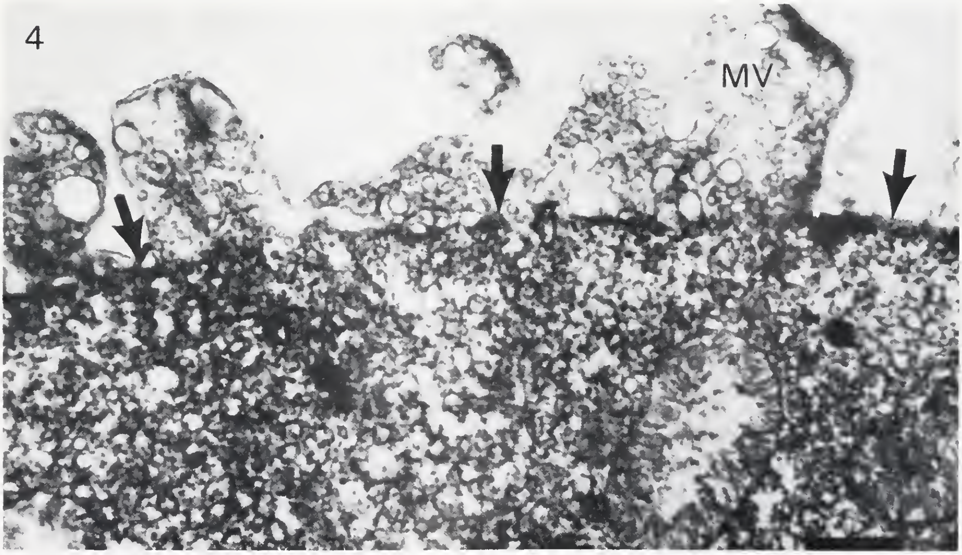


FIGURE 4. A transmission electron micrograph of the myoplasmic region of a *Ciona intestinalis* egg showing stout microvilli (MV) and an electron-dense layer immediately under the plasma membrane (arrows) that may represent the PML in section. From Sawada and Osanai (1981).

ooplasmic segregation can be explained by movements of extracellular elements associated with the plasma membrane. Reasonable candidates for these elements would be carbohydrate moeities associated with integral or peripheral membrane proteins.

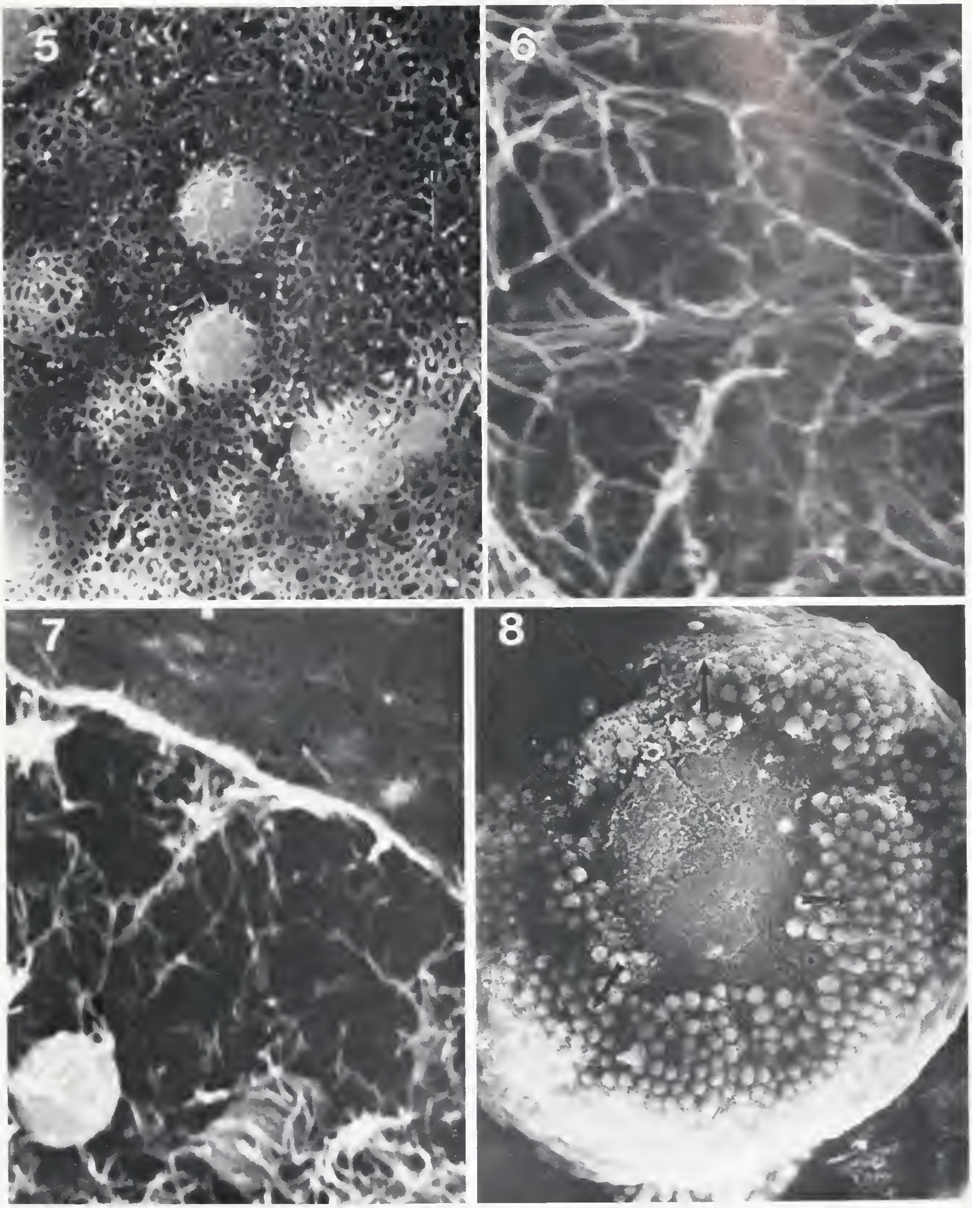
If plasma membrane proteins or other cell surface constituents translocate into the vegetal hemisphere during ooplasmic segregation and are not rapidly replaced, the lipid bilayer might be expected to be depleted of these components. In agreement with this prediction, Sawada (1983) points out that *Pyura michaelseni* eggs having recently completed segregation exhibit very fragile animal hemisphere surfaces that are the first parts of the egg to be ruptured by detergents or even by the light of a microscope lamp. This observation suggests that a local weakening of the egg surface in the animal hemisphere may be elicited by the depletion of surface components during ooplasmic segregation.

OOPLASMIC SEGREGATION OF CYTOSKELETAL SYSTEMS

Dynamic changes occur in the cortex of many eggs during early development (see Vacquier, 1981 for review). Among the best known of these changes is the elaboration of a cortical actin cytoskeleton shortly after fertilization in sea urchin eggs (Spudich and Spudich, 1979; Wang and Taylor, 1979). There is reason to believe that cytoskeletal systems may also be elaborated in the more internal regions of sea urchin eggs after fertilization (Moon *et al.*, 1983). The participation of cortical and internal cytoskeletal elements in ooplasmic segregation has recently been demonstrated by Jeffery and Meier (1983) in *Styela* and *Boltenia* eggs. Treatment of these eggs with the non-ionic detergent Triton X-100 solubilizes most of the cellular lipids, carbohydrates, proteins, and RNA, but does not efficiently extract a group of major proteins which comprise the egg cytoskeleton. Actin and a few proteins that show similarity to intermediate filament components are among these detergent-insoluble cytoskeletal proteins. Detergent extraction also reveals an intricate cytoskeletal framework which includes some of the organelles of the egg cytoplasm. The ectoplasmic, endoplasmic, and myoplasmic regions of the egg are each represented as a special cytoskeletal domain in the detergent-insoluble residue. The different cytoskeletal domains of the egg probably represent the modern equilvalent of what early embryologists referred to as the cytoplasmic ground substance (Wilson, 1925).

The myoplasmic cytoskeletal domain has been investigated most thoroughly to date, since it is present near the egg surface throughout early development and its features can be examined by scanning electron microscopy (SEM). The myoplasmic cytoskeletal domain consists of two parts. The surface of the domain is composed of a filamentous network called the plasma membrane lamina (PML) (Fig. 5). SEM shows that the PML is arranged in a two-dimensional plane immediately below the plasma membrane of *Styela* and *Boltenia* eggs. Sawada and Osanai (1981) have seen a thin electron-dense layer below the plasma membrane in the myoplasm of *C. intestinalis* eggs by transmission electron microscopy which is likely to be analogous to the PML of *Styela* and *Boltenia* eggs (Fig. 4). Actin is a major constituent of the PML since this structure completely disappears when eggs are detergent extracted in the presence of DNase I (Jeffery and Meier, 1983), an agent known to specifically depolymerize F-actin (Hitchcock *et al.*, 1976; Raju *et al.*, 1978).

Interior to the PML the myoplasmic cytoskeletal domain consists of a three-dimensional lattice of anatomosing filaments coursed by filament bundles (Fig. 6). The deep filamentous lattice (DFL), as this structure is called, is resistant to treatment with DNase I, colchicine, or low temperatures (Jeffery and Meier, 1983; Jeffery, unpub.). Consequently, microfilaments and microtubules are unlikely to be major constituents. The DFL may consist of intermediate filaments, but this point has not



FIGURES 5–8. SEMs of the myoplasmic cytoskeletal domain of Triton X-100-extracted *Styela plicata* and *Boltenia villosa* eggs.

FIGURE 5. The PML in a *B. villosa* egg at the conclusion of the first phase of ooplasmic segregation. Note the presence of the underlying pigment granules. ×10,000.

FIGURE 6. The DFL that underlies the PML in a *S. plicata* egg. Note the filamentous lattice coursed by a filament bundle. The lattice also contains the pigment granules (not shown). ×20,000.

FIGURE 7. The PML (above) and the underlying DFL (below) viewed from the side in a *S. plicata* egg. Note the association of filaments in the lattice with both pigment granules (lower left) and the PML. ×15,000.

FIGURE 8. The myoplasmic cytoskeletal domain as seen from the animal pole of a *B. villosa* egg early during the process of ooplasmic segregation. The PML is in the process of receding into the vegetal hemisphere (arrows) ×1200. From Jeffery and Meier (1983 and unpub.) and Jeffery (1983b).

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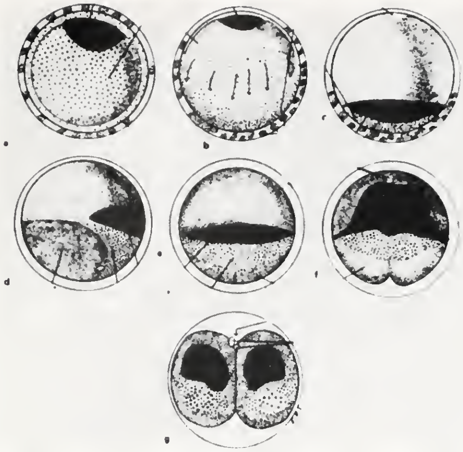
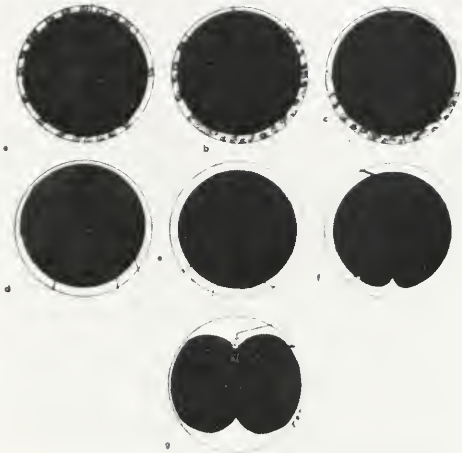
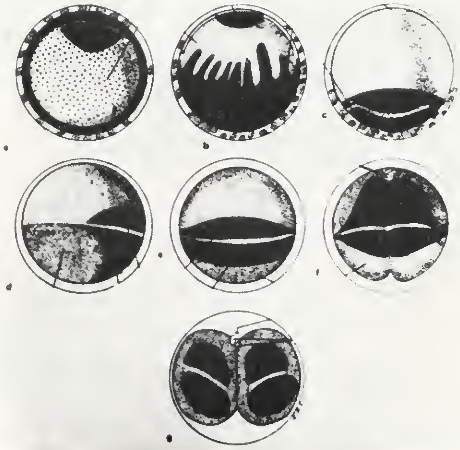
A$^+$



Histone



Actin



been established with certainty. Jeffery and Meier (1983) have shown that filaments of the DFL are attached to the PML, to the surrounding pigment granules (these also remain after extraction), and possibly to the adjacent cytoskeletal domains (Fig. 7).

Is the myoplasmic cytoskeletal domain of ascidian eggs elaborated after fertilization, like the cortical cytoskeleton of sea urchin eggs, or is it present in unfertilized egg and segregated into the vegetal hemisphere in concert with the other myoplasmic organelles? Two lines of evidence suggest that the myoplasmic cytoskeletal domain is already present in the unfertilized eggs of *Styela, Botenia,* and *C. intestinalis.* First, SEM indicates that the PML and the underlying DFL are present throughout the cortex of unfertilized eggs. Along with the myoplasmic pigment granules, the PML and DFL progressively recede into the vegetal hemisphere after fertilization (Fig. 8). During this recession, a 2–3 fold reduction appears in the length of spaces between the filaments of the PML (Jeffery and Meier, 1983). This suggests that the PML contracts during ooplasmic segregation. Second, staining of the PML with NBD-phallacidin, a mushroom derivative that specifically reacts with F-actin filaments (Barak *et al.,* 1980), also shows that this structure is originally present throughout the periphery of unfertilized eggs and is progressively restricted to the vegetal pole region during ooplasmic segregation (Jeffery and Meier, 1983; Sawada, 1983). These results indicate that the myoplasmic cytoskeletal domain is present before fertilization and imply that it is rearranged in concert with cytoplasmic organelles and egg surface components during ooplasmic segregation.

Ooplasmic Segregation of Messenger RNA

It has been known for some time that RNA is not uniformly distributed between the various cytoplasmic regions of ascidian eggs. Histochemical studies conducted with the eggs and embryos of *C. intestinalis* (Mancuso, 1959) and *Ascidia nigra* (Cowden and Markert, 1961) showed RNA staining primarily in the ectoplasm and, to a lesser extent, in the myoplasm. The maintenance of the characteristic staining properties of these regions during ooplasmic segregation implies that RNA molecules are also subject to cytoplasmic rearrangement. The RNA type detected in these studies was undoubtedly ribosomal RNA—the most prevalent type of RNA in the egg. The segregation of ribosomal RNA is perhaps not surprising because many ribosomes are associated with intracellular membrane systems that are rearranged along with the larger cytoplasmic organelles after fertilization (Berg and Humphreys, 1960).

Histochemical staining of RNA does not shed light on the behavior of maternal mRNA, a molecule that has been considered as a candidate for a morphogenetic determinant (Whittaker, 1977; see Jeffery, 1983a for review) and that is relatively rare in the egg. The mapping of maternal mRNA distributions has recently become

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FIGURE 9. A summary diagram of maternal mRNA distribution in the three cytoplasmic regions of *Styela plicata* eggs and embryos during early development. The dark areas represent the ooplasm(s) most concentrated in the respective RNA sequences. A. Poly(A)$^+$RNA is concentrated in the ectoplasm. B. Histone mRNA is uniformly distributed between ectoplasm, myoplasm, and endoplasm. C. Actin mRNA is concentrated in the ectoplasm and myoplasm. The stages shown in frames A–C of this summary are (from left to right and top to bottom): unfertilized egg; fertilized egg during ooplasmic segregation; the end of the first phase of ooplasmic segregation, fertilized egg during second phase of ooplasmic segregation; end of the second phase of ooplasmic segregation; first cleavage; and two cell embryo. The last three stages are viewed from the future posterior region of the embryo. The data used to construct this diagram are taken from Jeffery and Capco (1978) and Jeffery *et al.* (1983). The diagram was adapted from Conklin (1905).

possible due to the advent of *in situ* hybridization methods for use with sectioned eggs and embryos (Capco and Jeffery, 1978). The results of studies on the distribution of general poly(A)$^+$RNA, actin mRNA, and histone mRNA during early development of *Styela plicata* are summarized in Figure 9. Using *in situ* hybridization with a poly(U) probe (Jeffery and Capco, 1978), maternal poly(A) [presumably poly(A)$^+$RNA] was found to be significantly enriched in the ectoplasm relative to the other cytoplasmic regions of unfertilized eggs (Fig. 9A). High levels of poly(A) persist in the ectoplasm during ooplasmic segregation, when this region migrates extensively through the egg and eventually comes to rest in the animal hemisphere. The distribution of histone and actin mRNA has been investigated during ooplasmic segregation by *in situ* hybridization with the appropriate cloned DNA probes (Jeffery *et al.*, 1983). Histone mRNA is uniformally distributed among the cytoplasmic regions of the egg, but actin mRNA is highly concentrated in the ectoplasm and the myoplasm (Fig. 9B–C). As in the case of poly(A)$^+$RNA, histone and actin mRNA segregate with the ooplasms in which they were originally localized before fertilization. These results imply that maternal mRNA molecules localized in the various cytoplasmic regions of the egg also participate in ooplasmic segregation. If maternal mRNAs do serve as morphogenetic determinants, ooplasmic segregation would seem to promote their distribution to the proper embryonic cell lineages during cleavage.

COORDINATION OF OOPLASMIC SEGREGATION

We have seen that at least four different components of ascidian eggs; cytoplasmic organelles, cell surface elements, cytoskeletal domains, and maternal mRNA molecules, participate in ooplasmic segregation. It is possible that these components are rearranged in concert because they are associated with one another in a structural complex. In the succeeding paragraphs we will discuss the evidence for a complex of this kind, with the PML as its central element.

Morphological studies indicate that the PML is attached to the DFL (Fig. 7). The PML, through its interaction with the DFL, is also indirectly associated with the myoplasmic pigment granules, other myoplasmic organelles, and probably the ectoplasmic cytoskeletal domain (Jeffery and Meier, 1983). It seems likely (but yet to be demonstrated) that the PML may be associated with plasma membrane proteins, as are similar actin filamentous networks in somatic cells (Ben-Ze'ev *et al.*, 1979; Sheetz, 1979; Mesland *et al.*, 1981). If these membrane proteins span the lipid bilayer and interact with substances outside of the egg, the movement of test cells, surface carbohydrates, and particles that adhere to the egg surface could be coordinated with cytoskeletal and organellar rearrangements in the cytoplasm. The picture of myoplasmic organization that emerges is that of a complex containing the PML, plasma membrane proteins, and the DFL system.

The rearrangement of maternal mRNA may also be directed by an association with this complex. The interaction of mRNA with the cytoskeletal framework has been reported recently in a number of different types of eukaryotic cells (Lenk *et al.*, 1977; Cervera *et al.*, 1981; van Venrooij *et al.*, 1981; Jeffery, 1982b; Moon *et al.*, 1983). The possibility of mRNA-cytoskeletal interactions has recently been tested by subjecting sections of detergent extracted *S. plicata* eggs to *in situ* hybridization with poly (U) and cloned DNA probes (Jeffery, 1984). Most of the poly(A)$^+$RNA, histone mRNA, and actin mRNA was found in the cytoskeletal framework after detergent extraction. These molecules were also shown to be localized in the same regions of the extracted eggs as they were in intact eggs. Their localization was not altered when eggs were detergent extracted and subjected to *in situ* hybridization while the mRNA molecules were in flux during ooplasmic segregation. These results suggest that the association of mRNA molecules with the cytoskeletal framework is responsible for their behavior during ooplasmic segregation. The nature of the egg cytoskeletal elements linked to mRNA molecules is still unknown. It seems likely that microtubule or microfilament systems (*i.e.,* the PML) are not involved, however, since the mRNA-cytoskeletal associations are insensitive to low temperature, cytochalasin B, and DNase I treatment (Jeffery, 1984). At this time the best candidate for a mRNA binding site in the myoplasmic cytoskeletal domain is the DFL.

The information at hand suggests that cell surface elements, cytoskeletal systems, myoplasmic organelles, and maternal mRNA segregate coordinately due to their association with one another in a structural complex. The movement of this complex into the vegetal pole of the egg during ooplasmic segregation may be similar to capping in somatic cells (Zalokar, 1980), where a complex of cortical actin filaments and surface ligands migrate in unison to particular regions of the cell surface (Albertini and Anderson, 1977; Toh and Hard, 1977).

Mechanism of Ooplasmic Segregation

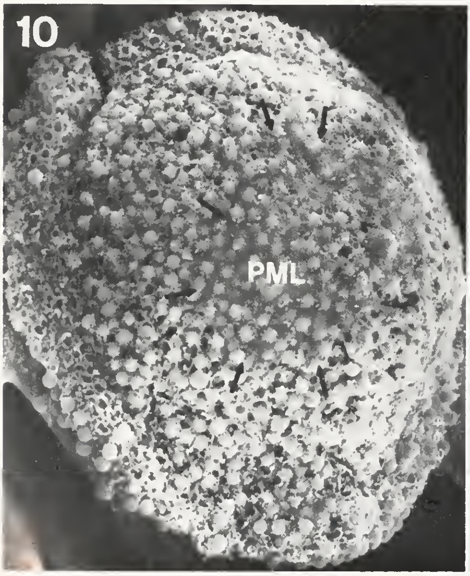
What is the nature of the motive force required to generate the concerted movements of ooplasmic segregation? This force is likely to be generated by one of the components of the complex that moves into the vegetal hemisphere. A number of different studies suggest, however, that neither egg surface components nor internal cytoplasmic structures are responsible for ooplasmic segregation. When the lectin binding sites of *Phallusia mammillata* eggs were immobilized by attaching them to nylon sheets containing concanavalin A, the myoplasm was segregated into the vegetal region of the egg as usual after fertilization (Zalokar, 1979). This suggests that the migration of plasma membrane ligands does not drive ooplasmic segregation. When eggs were centrifuged to stratify the internal cytoplasmic components (Conklin, 1931), they still went through the shape changes characteristic of ooplasmic segregation (Sawada, 1983), they were able to move applied carmine particles into their vegetal hemispheres (Sawada, 1983), and the PML receded into the vegetal hemisphere (Fig. 10) after fertilization (Jeffery and Meier, 1984). These results suggest that the association of the DFL and attached cytoplasmic organelles with the PML is not required for ooplasmic segregation.

A number of lines of evidence suggest that the contraction of the PML drives the first phase of ooplasmic segregation. As noted above, the filamentous network of the PML has been observed by SEM to gradually recede into the vegetal hemisphere of the egg, with a concomitant shortening of its interstices, during ooplasmic segregation (Jeffery and Meier, 1983). As also pointed out earlier, it is very likely that the PML is composed of F-actin, since it disappears when eggs are extracted with detergent in the presence of DNase I (Jeffery and Meier, 1983) and it is stained by NBD-phallacidin (Jeffery and Meier, 1983; Sawada, 1983). The involvement of a microfilament system in the first phase of ooplasmic segregation is also supported by the sensitivity of this process to cytochalasin B (Zalokar, 1974; Reverberi, 1975; Sawada and Osanai, 1981). Microtubules, however, do not seem to be involved since there is no sensitivity to colchicine or vinblastine (Zalokar, 1974; Jeffery, unpub.).

Despite the availability of good evidence that the motive force for ooplasmic segregation involves a contractile system containing microfilaments, it must be admitted that filaments of this kind have never been positively identified in the cortex of ascidian eggs. It has been suggested by Reverberi (1975) that putative cortical microfilaments are too sparse to be detected in thin sections. It also seems possible that microfilaments could be destroyed by the procedures that have been used to fix the eggs. Recently, however, Sawada (1983) has reported very sparse, 10–15 nm filaments in whole mounts of cortices isolated from *C. intestinalis* eggs that may be the elusive microfilaments.

Jeffery and Meier (1983; also see Jeffery, 1983b) and Sawada (1983) have proposed very similar models for the mechanism of ooplasmic segregation in ascidian eggs. These models are based on the contraction of an actin-containing, PML-like structure, and the association of this structure with the other elements of the egg in a structural complex that participates in ooplasmic segregation after fertilization. The Jeffery and Meier model proposes that the PML is connected to the egg plasma membrane and to more internal cytoplasmic constituents (the pigment granules, other myoplasmic organelles, the ectoplasmic cytoskeletal domain, and maternal mRNA) via the DFL. The latter connections are suggested by the observations discussed in previous sections of this article and by the direct observations of Sawada (1983) indicating that the sub-cortical myoplasm moves downward as a gelatinous mass which becomes folded, as if it were dragged by another structure (presumably the PML) (Fig. 11), during ooplasmic segregation.

According to the Jeffery and Meier model (Fig. 12), the initial event of ooplasmic segregation is a contraction of the PML. The PML is proposed to gradually shorten as ooplasmic segregation proceeds, pulling the myoplasmic domain with it on its inner surface and integral plasma membrane proteins with it on its outer surface. The accumulation of excess membrane proteins at the vegetal pole during the con-



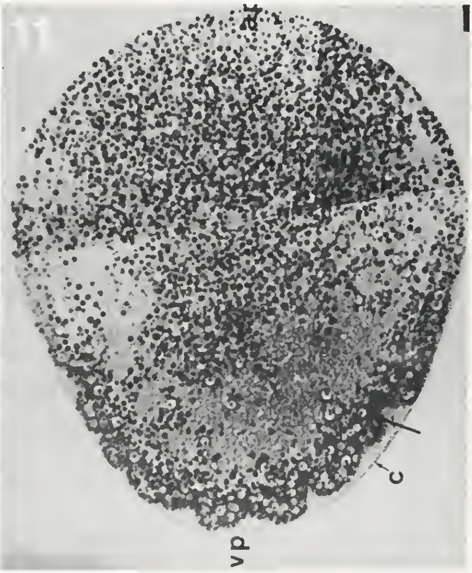


FIGURE 10. SEM of the PML of a *Boltenia villosa* egg that was centrifuged prior to fertilization to displace the DFL and associated pigment granules from the myoplasmic cytoskeletal domain and then extracted with Triton X-100 about 15 minutes after insemination. Note that the PML (outlined by arrows) has receded to a position at one end of the egg as is usual during ooplasmic segregation, but the pigment granules and associated filaments are dispersed throughout the egg. ×1200. From Jeffery and Meier (1984).

FIGURE 11. A thin section of a *Ciona intestinalis* egg undergoing the first part of ooplasmic segregation showing the buckling of the cortical cytoplasm under the plasma membrane (and presumably the PML). The bar is 10 microns. From Sawada (1983).

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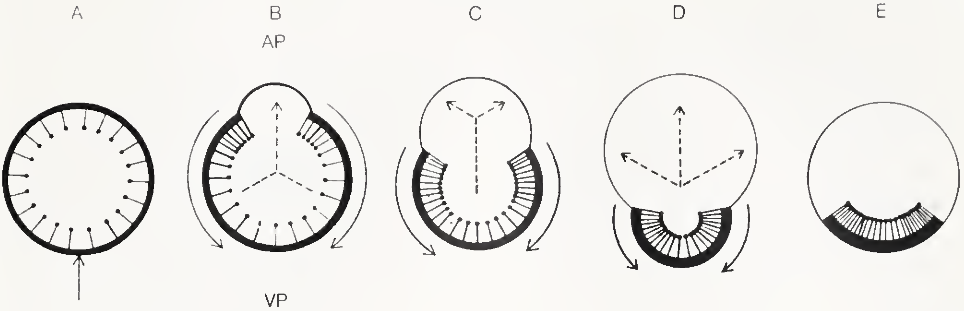

FIGURE 12. The Jeffery and Meier model for the first phase of ooplasmic segregation. A. An unfertilized egg. The vertical arrow indicates the focal point for segregation. B–D. Fertilized eggs in the process of ooplasmic segregation. The arrows represent the directions of ooplasmic movement: arrows with solid lines for the myoplasm and arrows with broken lines for the endoplasm. E. A zygote which has completed the first phase of ooplasmic segregation. In each diagram the thick egg boundaries represent parts of the plasma membrane with PML underneath, and the thin egg boundaries represent parts of the plasma membrane without the PML. The structures attached to the inside of the PML in the myoplasm represent the filaments of the DFL and its associated components, pigment granules and localized mRNA molecules of the myoplasmic cytoskeletal domain. AP, animal pole; VP, vegetal pole. From Jeffery and Meier (1983).

traction of the PML may be the reason for restriction of microvilli to this region (Fig. 4) (Sawada and Osanai, 1981). The contraction of the PML into the vegetal hemisphere is also proposed as the force that displaces most of the endoplasmic yolk platelets into the animal hemisphere. The PML is thought to form a contractile ring at its upper margin, and this ring coupled with a weakening of the plasma membrane (possibly due to the depletion of integral membrane proteins), would lead to the bulging of endoplasm in the animal hemisphere. Further tightening of the contractile ring, shortening of the PML, and endoplasmic bulging in the animal hemisphere could also make the vegetal hemisphere appear to protrude a cytoplasmic lobe (Fig. 7), as is usually seen during ooplasmic segregation (Fig. 3).

A significant question which arises from the models is whether the force generating the movement of the PML is an active, energy-dependent process, such as that based on an actomyosin system, or the recoil of a previously stretched elastic system. Treatment of ascidian eggs with ouabain, a specific inhibitor of the Na^+K^+–ATPase, or dinitrophenol, an uncoupler of oxidative phosphorylation, does not affect ooplasmic segregation, suggesting that it may not be energy dependent (Jeffery, unpub.). There is some question concerning the effect of these substances on the energetics of ascidian eggs, however, and at this time the problem of energy dependence remains open.

A way in which the Jeffery and Meier model and the Sawada model differ is that the former proposes that the complex containing the PML exists around the entire periphery of the unfertilized egg, while the latter suggests that a defect in the complex exists at the animal pole (so that the complex appears like a basket covering the lower portion of the unfertilized egg; Fig. 13A). A local break point in the PML at the animal pole of the egg must be postulated in the Jeffery and Meier model to allow the complex to contract into the vegetal hemisphere as a unit. This break point is already present in the contractile basket model of Sawada. The Sawada version is supported by observations on *C. intestinalis* (Reverberi, 1956), *Ascidiella scabra* (Dalcq and Vandebroek, 1937), and *Phallusia mammillata* (Zalokar, 1974) eggs, each of

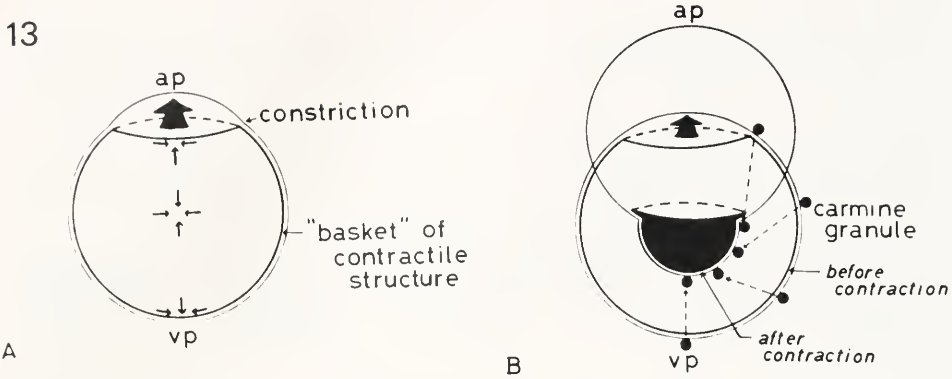

FIGURE 13. The Sawada model for the first phase of ooplasmic segregation. A. A diagram showing the contractile basket structure with a mouth near the animal pole (ap) and contracting in all directions at all points (small arrows). This contraction pushes the inner cytoplasm toward the animal pole (large arrows) making a constriction at the mouth of the basket. B. A diagram depicting the contraction of the basket into the vegetal hemisphere. The inner cytoplasm is pushed out toward the animal pole. Carmine granules are moved toward the vegetal pole (vp) as a result of the movement of surface elements dragged vegetally by the contracting basket. From Sawada (1983).

which actually show a defect in the myoplasm at the animal pole. In fact, in many instances the myoplasm is present in only the lower two-thirds of the unfertilized egg in these species, leaving a broad ectoplasmic cap in the animal hemisphere (Reverberi, 1956). Such a defect at the animal pole has not been seen by SEM or light microscopy in *Styela* or *Boltenia* eggs (Jeffery, unpub.), however, it may be too small to be detected. It is also possible that the myoplasm is thinner in the animal hemisphere than in the vegetal hemisphere of these eggs and that the myoplasmic break point is formed *de novo* after fertilization. These differences in the structure of the myoplasm could lead to variations in the manner of ooplasmic segregation between different families of ascidians.

Although the models discussed in the preceeding paragraphs provide a working hypothesis in which experiments can be devised to further understand the mechanism of the first phase of ooplasmic segregation, they do not provide insight into the mechanism of the second step in this process: the formation of the myoplasmic crescent. Unfortunately, there has been little study of the extension of the myoplasm into a crescent and the return of the ectoplasm and endoplasm to their original positions in the egg. It does seem likely, however, that the microtubular systems of the asters play an important role in these events.

CONTROL OF OOPLASMIC SEGREGATION

Cellular mechanisms must exist that regulate when and where ooplasmic segregation takes place in ascidian eggs. It is likely that such regulatory events are initiated by fertilization. A pulse of calcium, produced by an influx of ions into the fluid cytoplasm from the surrounding environment or from intracellular depots, is known to occur at the time of sperm entry in a number of different eggs (see Jaffe, 1983 for review). This transient rise in free calcium is thought to trigger the processes related to egg activation, which includes ooplasmic segregation in eggs of the brown alga *Fucus* (Jaffe and Nuccitelli, 1977). A transient rise in free calcium also appears to induce egg activation and ooplasmic segregation in ascidians, since treatment of unfertilized *C. intestinalis* (Steinhardt *et al.,* 1974), *Ascidia malaca* (Bevan *et al.,* 1977), *Styela* (Jeffery, unpub.), and *Boltenia villosa* (Jeffery, 1982a) eggs with the divalent ionophore A23187 causes egg activation. In eggs of *Boltenia villosa,* where the process of ooplasmic segregation can be followed very precisely due to high contrast between the brilliant orange myoplasm and the white endoplasm (Jeffery, 1982a), ionophore A23187 promotes polar body formation, the streaming of myoplasm and ectoplasm into one hemisphere of the egg, egg surface deformations, and the development of a patch of orange pigment reminiscent of the myoplasmic crescent of fertilized eggs. The patch of myoplasm contains mitochondria as well as pigment granules and cannot be distinguished from a natural myoplasmic crescent by electron microscopy (Jeffery, 1982a). A cleavage furrow begins to form in many of the ionophore-treated eggs temporarily splitting the orange pigmented area, but then recedes, presumably because of the absence of a sperm aster.

It is likely that the induction of ooplasmic segregation in ascidian eggs by ionophore A23187 is the consequence of a rise in intracellular calcium, since it can be mimicked by high external concentrations of calcium or by theophylline, a substance thought to increase the intracellular calcium concentration (Bevan *et al.,* 1977). It is also inhibited by the calcium antagonist, lanthanum (Bevan *et al.,* 1977). The rise in calcium must be due to the opening of intracellular calcium depots, rather than to the entry of calcium from the surrounding environment, since the action of ionophore cannot be blocked by removing extracellular calcium from the sea water. These results suggest that a release of calcium from the intracellular depots triggers ooplasmic segregation at the time of fertilization. The calcium flux is probably mediated by the fusion of the sperm with the mature egg. The intracellular calcium depots and the cellular machinery necessary for ooplasmic segregation must be functional as early as the primary oocyte stage, however, since ovarian eggs containing intact GVs can also be induced to undergo a segregation of myoplasm by treatment with ionophore A23187 (Jeffery, 1982a).

Although a rise in intracellular calcium appears to control the timing of ooplasmic segregation, this does not explain how the directional properties of segregation are regulated. There are two major explanations for the formation of a single focal point for ooplasmic segregation. First, a rise in calcium anywhere in the cell may trigger an episode of ooplasmic segregation that is spatially directed along the animal-vegetal axis by a pre-existing determinant of egg polarity. Second, a focal point for ooplasmic segregation may be generated either toward or away from the localized region of calcium flux itself. These possibilities were tested by aligning unfertilized *Boltenia villosa* eggs against a small glass rod coated with ionophore A23187, such that only a single point on the egg surface was exposed to the antibiotic (Jeffery, 1982a). Robinson and Cone (1980) have shown that the ionophore, a substance highly insoluble in sea water, is slowly emitted from the rod forming a steep concentration gradient. The portion of the egg touching the rod was exposed to the highest concentration of ionophore and consequently would be predicted to exhibit the highest potential to release calcium from any nearby storage site. The majority of the eggs aligned against the rod in this fashion showed a segregation of orange myoplasm toward the point of contact (Fig. 14A). This was not an effect of the glass rod itself because unfertilized eggs aligned against untreated rods and then exposed to ionophore A23187 showed random foci of ooplasmic segregation with respect to the point of contact (Fig. 14B). The results suggest that the direction of ooplasmic segregation is not dictated by a pre-existing determinant of polarity in the egg, but that it is focussed toward the region of the egg cytoplasm exposed to the highest levels of calcium. It is presumed that the local rise in calcium is responsible, either directly or indirectly, for the

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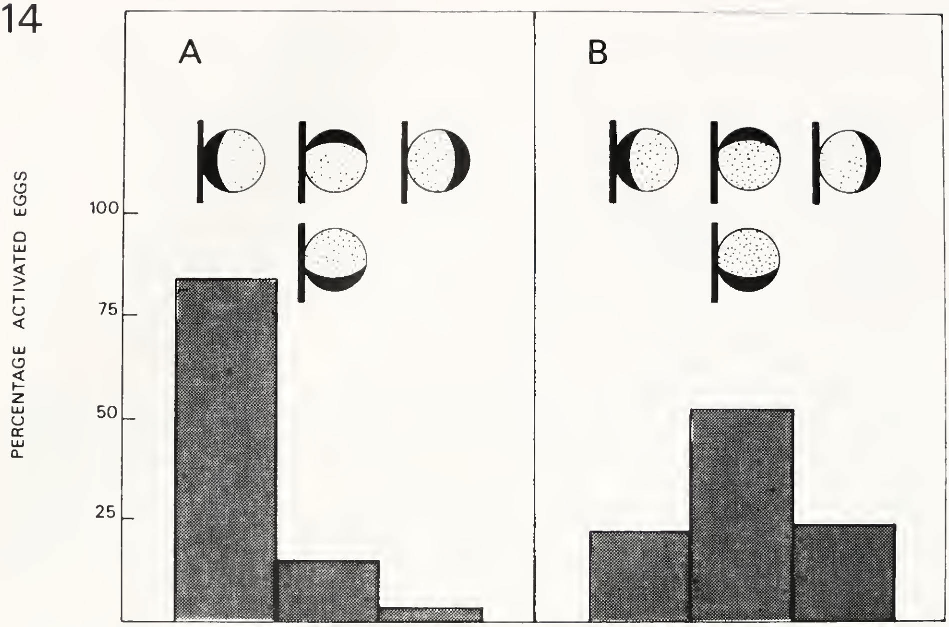

FIGURE 14. The focal points for the myoplasm in ionophore A23187-activated *Boltenia villosa* eggs that have completed ooplasmic segregation aligned against glass rods. A. The direction of myoplasmic polarization in eggs aligned against an ionophore-coated rod. Most eggs form myoplasmic caps on the side nearest the rod. B. The direction of myoplasmic polarization in eggs aligned against an untreated rod. Ionophore A23187 was present in the surrounding sea water during the experiment depicted in B. The myoplasmic caps form at random positions in the eggs with respect to the rod. From Jeffery (1983b).

contraction of the PML, which would contract toward the highest point of an intracellular calcium gradient formed in the egg. The nature of the mechanism through which calcium initiates PML contraction and the physical parameters actually responsible for the movement of the PML, however, remain to be explored.

The experiments involving a localized application of ionophore A23187 cited above also imply that many points along the circumference of the unfertilized egg are able to serve as focal points for ooplasmic segregation. Two other observations also support this idea. First, if unfertilized *Boltenia villosa* eggs are simultaneously aligned against two rods coated with ionophore A23187, many of these form double foci of ooplasmic segregation, each of which is centered near the point on the egg surface closest to the rod (Jeffery, 1982a). Second, if unfertilized *Styela* eggs are cut into as many as three fragments, each of these fragments are capable of completing ooplasmic segregation when treated with ionophore (W. R. Bates, pers. comm.). Even fragments containing less than 10% of the original volume of the egg are able to segregate myoplasm. At first these results seem to contradict the observation of Conklin (1905) that ooplasmic segregation in *Styela* eggs is focussed toward the vegetal pole. The results can be reconciled, however. The egg could be totipotent for the formation of a focal point for segregation, but under normal conditions sperm may only enter the egg and cause a local flux of calcium somewhere in the vegetal hemisphere. The problem of the spatial control of ooplasmic segregation and the subsequent fixation of cytoplasmic patterns essential for early development in ascidian eggs seems to be reduced to a single important question. Why does the sperm always enter the egg in the vegetal hemisphere? At present there is no answer to this question. Micropyles have never been described in ascidian eggs. Indeed, the issue of a pre-determined site for sperm entry *per se* in ascidian eggs is yet to be rigorously demonstrated.

CONCLUDING REMARKS

This article has presented a review of some new information on ooplasmic segregation and its role in the generation of developmental patterns in ascidian eggs. Although we have addressed only one of the many groups of organisms that are known to exhibit ooplasmic segregation during early development, it is felt that events similar to those that occur in ascidian eggs may also take place in other species, albeit less spectacularly. For instance, a number of observations suggest that microfilaments may also be responsible for ooplasmic segregation in other kinds of eggs. Ooplasmic segregation in zebrafish eggs (Katoh, 1983), the deformation movements and rearrangement of ooplasmic constituents in *Tubifex* eggs (Shimizu, 1982), and the segregation of germ line granules in *Caenorhabditis elegans* eggs (Strome and Wood, 1983) have been shown to be sensitive to cytochalasin B. A microfilamentous network has actually been demonstrated in the cortex of *Tubifex* eggs (Shimuzu, 1982), sea urchin eggs (Spudich and Spudich, 1979; Wang and Taylor, 1979), and frog oocytes and eggs (Franke *et al.*, 1976; Gall *et al.*, 1983; Franz *et al.*, 1983). Moreover, *Xenopus laevis* eggs have recently been shown to contain a lattice of intermediate filaments interior to the cortical actin cytoskeleton (Gall *et al.*, 1983; Franz *et al.*, 1983), which is highly reminiscent of the spatial relationship between the DFL and PML in ascidian eggs.

The temporal and spatial aspects of ooplasmic segregation may also be regulated by a local calcium flux in many eggs. Ionophore A23187 promotes ooplasmic segregation in the eggs of *Fucus* (Robinson and Cone, 1980), *Tubifex* (Shimizu, 1978), and *Xenopus* (Schroeder and Strickland, 1974), and in some of these systems segregation is accompanied by the contraction of the egg cortex (Elinson, 1975; Shimuzu, 1979; Merriam and Sauterer, 1983). There are even some data, besides those already discussed for ascidians, that more than one potential focal point for ooplasmic segregation can exist in an egg. In the egg of the Medaka fish, *Oryzias laptipes,* the cortical cytoplasm normally streams toward the point of sperm entry at the animal pole and large oil droplets simultaneously flow toward the vegetal pole after fertilization. Sperm enter the egg via a micropyle in the chorion over the animal pole. If the egg is cut into two fragments, ooplasmic segregation proceeds in each fragment (Sakai, 1964). The cytoplasm streams toward the point of sperm entry in the animal fragment. If the vegetal fragment (which has no micropyle) is prick activated, however, the cortical cytoplasm usually streams toward and the oil droplets away from the site of pricking. Prick activation at two sites on the egg surface can lead to two foci of cortical streaming just as multiple sites of ionophore application can cause two myoplasmic caps to form in ascidian eggs.

Clearly, much new information on ooplasmic segregation has become available since the last review of this process was published (Costello, 1948). As more information on ooplasmic segregation is accumulated in ascidian eggs and other embryonic systems, it may be possible to develop a general hypothesis for the generation of egg patterns by cytoplasmic movements during early development.

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STRUCTURE AND FUNCTION OF THE TERMINAL ABDOMINAL APPENDAGES (PYGYPODIA) OF PHOTURID FIREFLY LARVAE

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ABSTRACT

The morphology of the pygypodia, the eversable terminal appendages of the abdomens of photurid firefly larvae, was studied with particular reference to the integumental and muscle systems. The eversable nature and observed versatility of these appendages apparently result from the antagonistic action of pygypodia-associated muscles and those muscles which control (pseudo)coelomic fluid pressure in the abdomen, as well as from several fine structural features of the integument. Continuous sheets of parallel microfibrils in the endocuticle form a tough elastic inner sheath within each pygypodium. The integument bears rows of spurs, whose interconnection by the microfibril sheets may aid in their positioning as well as in their orderly and consistent packing during inversion and eversion of the pygypodia. The loose organization, irregularity of shape, and sparsity of sites of attachment of the epidermal cells, relative to the basement membrane, may all contribute to the flexible, yet strong, inner structure of the integument.

INTRODUCTION

Much is known about the anatomy and function of typical larval insect abdominal segments and their appendages (see Snodgrass, 1935, for review). There is little information, however, about less usual appendages such as the eversable pygypodia (Snodgrass, 1935) on the terminal abdominal segments of photurid firefly larvae. These appendages are extremely versatile in enabling the animal to attach to and move about on a variety of substrates. Their versatility and relatively simple structure makes this an interesting system, both in its basic morphology and in the opportunity to relate this morphology to the observed functions.

This paper describes the gross anatomy and the fine structure of these photurid pygypodia with particular reference to the integument and muscle systems. We also consider the functions of these appendages and speculate on their possible evolutionary origins.

MATERIALS AND METHODS

Photuris larvae were collected in Guilderland (near Albany, New York) during May and July and were stored cold and damp, in a controlled light/dark regime (McLean *et al.*, 1972). The larvae were fed crushed snails and were maintained in an intermolt state.

A variety of light and electron microscopical techniques, including bright field, phase, and Nomarski differential interference microscopy, and transmission and scanning electron microscopy, were used to observe dissected specimens and whole mounts of the pygypodia. To maintain the pygypodia partially or completely everted, larvae

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were injected with water-insoluble Vinyl Acetate Red (Ward's Natural Science Establishment). This compound polymerizes into a rubbery mass which plugs the pygypodia and keeps them from collapsing or being retracted.

For transmission electron microscopy (TEM), individual pygypodia were dissected out and immediately fixed (two hours; 4°C in 0.1 *M* cacodylate-buffered 3% glutaraldehyde at pH 7.2). The tissues were rinsed in buffer and post-fixed overnight (4°C in 1% osmium tetroxide in 0.1 *M* cacodylate buffer). All tissues were dehydrated in a graded ethanol series and were embedded in Spurr low viscosity resin (Spurr, 1969). Thin sections were cut with glass knives or with a Dupont diamond knife on an LKB Ultratome III. The sections were mounted on 100 or 200 mesh uncoated grids and were stained for 25 minutes with saturated uranyl acetate in 50% methanol at 37°C, and for five minutes at room temperature in lead citrate (Reynolds, 1963).

The specimens were examined at 60 kV with an AEI EM6B transmission electron microscope, which was calibrated by a #10002X crossed-line grating replica calibration grid (E. F. Fullam, Inc.).

To study cuticular elements, pygypodia were split longitudinally, dissected out, and placed for a week in a 10% solution of aqueous potassium hydroxide, which dissolves the cellular elements and extracts some of the cuticular structures, but leaves the exocuticle essentially intact.

For scanning electron microscopic (SEM) observation, pygypodia were everted by the vinyl acetate treatment, air dried, and then sputter-coated with gold and observed in an ISI Super Mini II scanning electron microscope.

RESULTS

An undisturbed larva typically rests with its pygypodia everted or extended (Fig. 1). When the animal walks, it inverts or retracts the entire pygypodial apparatus, using the blunt end of the abdomen as an "appendage" accessory to the legs. In this way, the abdomen both supports the body and provides a point of anchorage or adhesion against which the rest of the animal's locomotory apparatus can work.

Under special conditions, *i.e.*, hanging from an edge or righting itself when it has been turned on its back, the animal everts the pygypodia, which then provide a stable site of attachment and free the rest of the body to explore or to otherwise respond to the particular situation. This attachment is equally effective on rough (*e.g.*, paper, soil, etc.) or smooth (*e.g.*, glass or plastic) substrates.

Control of pygypodial movement appears selective; not all the podia necessarily evert or invert at a given time, nor need all extend or retract to the same degree. All in all, the pygypodia are quite effective in accommodating to whatever substrates they encounter.

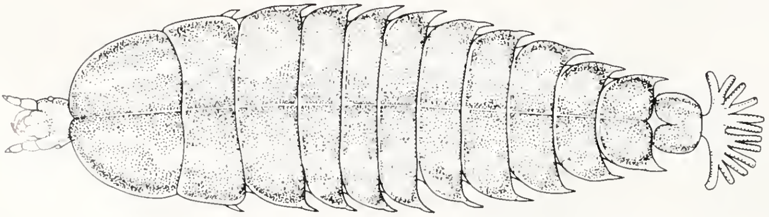


FIGURE 1. Dorsal view of a mature photurid larva. The everted pygypodia form the fan-like structure emerging from the rear.

External morphology

The pygypodial apparatus is located on, and can be withdrawn into, the ninth abdominal segment (Figs. 1, 2) and an extension thereof. Because of the small size of the extension, it is difficult to determine if it is actually a tenth abdominal segment. Dissection reveals that the extension appears to have lost the tergal plate, being essentially covered by the tergum of the ninth segment, but that it does have pleural plates and a small and indistinct sternum.

All pygypodia originate from a common basal stalk (Figs. 1, 2a) which is continuous with the flexible integument of this last abdominal segment, the extension of segment IX. The entire pygypodial apparatus exhibits bilateral symmetry; each side consists of eight individual podia, of which six are located dorsally and two ventrally. Of the dorsal six, the outer four originate from a common branch of the main stalk, whereas the remaining two come from a separate, more medial branch (Fig. 2a). Each ventral set of two pygypodia originates from yet another branch of the main stalk. Together, when everted, the twelve dorsal pygypodia display a fan-shaped pattern (Figs. 1, 2a), and the ventral pygypodia assume a smaller, but similar pattern.

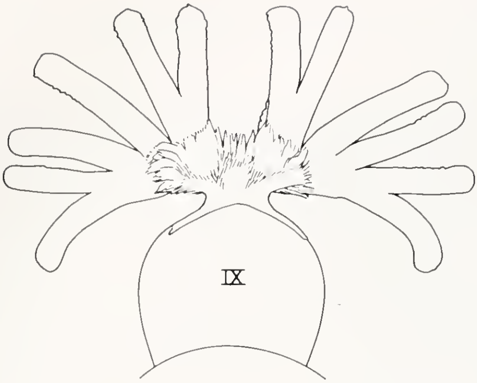
Individual pygypodia are cylindrical, and each has a longitudinal crease running dorsally along the entire length and a distinct protrusion or bulb at the tip (Fig. 4). The more medial podia are slightly longer than the lateral ones, and the ventral podia are shorter and stouter than their dorsal counterparts.

The pygypodia have on their surfaces sets of equidistantly-spaced transverse rows of cuticular outgrowths (Figs. 3–7). On the ventral side of each pygypodium these appear as spurs whose points, when everted, are oriented towards the animal's abdomen. Dorsally, these outgrowths are represented by smaller bi-, tri-, and quadri-cuspid barbs (Fig. 5).

Each abdominal segment up to the eighth has the typical arrangement of dorsal longitudinal muscles, *i.e.,* bilateral bundles of parallel longitudinal fibers running from one intersegmental fold to the next (Snodgrass, 1935). In segments eight and nine, this arrangement of the longitudinal muscles is modified (Figs. 2b, c). First, the muscle bundles float freely in the body cavity, with only a few of the fibers attaching at the eighth-ninth intersegmental fold. Second, as the fibers to the dorsal pygypodia proceed posteriorly within the eighth segment, their orientation pattern abruptly changes from a parallel to a twisted one, giving the muscle bundles an appearance similar to twine or spun yarn (Fig. 2b). Within the ninth segment, the tangle resolves itself into a series of smaller bundles, each of which is inserted directly into the inside tip of a dorsal pygypodium. The shorter, stouter ventral pygypodia present a completely different picture of muscle attachment (Fig. 2c). There are six muscle bands, three of which originate from the left and three from the right antecosta of the eighth segment. The bands from the left side insert respectively on the left lateral and the two central pygypodia, while the mirrored or opposite arrangement occurs on the right side. Thus the two central pygypodia each receive two muscle bands, one from either side.

Fine structure

The endocuticle of the integument appears vaguely lamellar because of the orientation of bundles of microfibrils (Fig. 8). The microfibrils form a continuous sheet which is relatively planar in the dorsal region of a pygypodium, but not in the ventral region, where the spurs are found. Here, the sheet forms a pattern of peaks and valleys (Fig. 8). The peaks are located beneath the spurs, while the valleys lie between them. Microfibrils from the peaks extend into the spurs while, conversely, microfibrils in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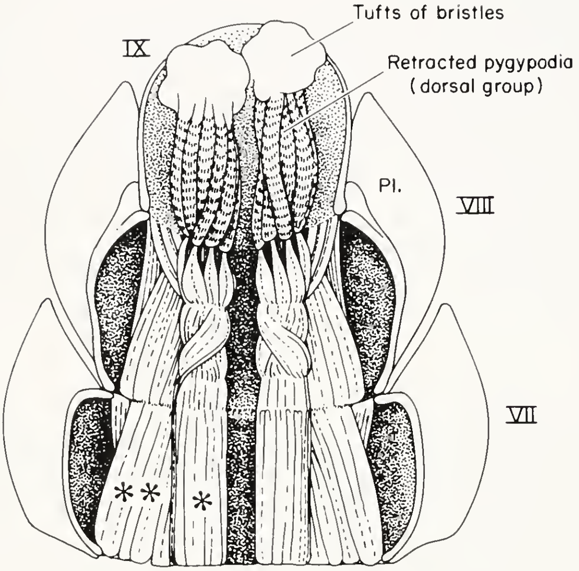
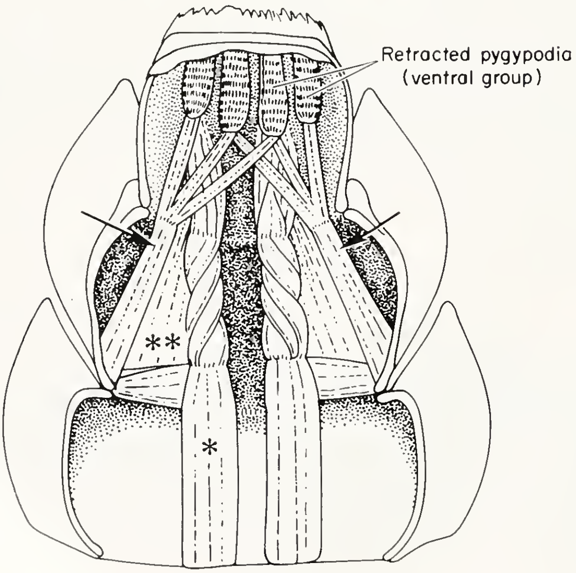

Tufts of bristles
IX
Retracted pygypodia
(dorsal group)
Pl.
VIII
VII
**
*


Retracted pygypodia
(ventral group)
**
*

the valley portions appear to be attached to the epidermis by relatively densely staining material which may represent adhesion plaques. The endocuticle beneath the microfibril sheet appears somewhat amorphous (Figs. 8, 9).

The epidermal cells are irregular in shape, loosely organized, and have interlocking borders (Figs. 8, 9). These borders are marked by extensive desmosomes (Fig. 8), and individual cells present numerous vacuoles and small spherical objects at the epidermal-cuticular interface; these may represent Golgi complexes or plasma membrane plaques which may be involved in cellular uptake or secretion (Locke, 1976).

Figures 10 and 11 present thin sections of single pygypodial muscle bands. With our fixation, the connective tissue sheaths around the muscle bands appear rippled. Transverse sections show the typical actin-myosin arrangement within some of the fibrils of the muscle bands (Fig. 11). The fibrils are not all arranged in parallel; thus, in a single transverse plane, only a small proportion of the cut fibrils present the typical "cross-section" actin and myosin patterning. These sections also show variability in the arrangement of the Z-line material (Fig. 11).

Figure 11 (inset) shows the hemidesmosomal attachments of the Z-line material of the pygypodial muscle to the plasma membrane and the basement membrane.

Operation of the pygypodia

Inversion. Inversion occurs when the contracting muscle fibers pull the pygypodial tip into the hollow of the pygypodium. This process diminishes the volume of the appendages and is therefore in opposition to the coelomic fluid pressure, which may contribute to the compression of the newly inverted integument. The dorsal crease may provide the pygypodium with an initial fold to guide the further packing of the integument and spurs. During this process, the latter are pried off the substrate, one row at a time, and are internalized and reoriented to fold properly and to intermesh compactly (Figs. 6, 7).

Eversion. There is no array of muscle fibers, comparable to those discussed above, whose action would directly evert the pygypodia. The lack of any obvious musculature for this purpose, together with the ease with which the pygypodia may be everted by increasing the coelomic pressure (as in pressing gently on the animal's abdomen), suggests that the eversion mechanism may be hydrostatic, and that the animal forces the podia to extend by tightening the abdominal musculature and driving hemolymph into them. During this process, the basement membranes of the inverted and everted portions of the pygypodia are in tight apposition (Fig. 9). This close contact produces a structure resembling a movable plug (the still-inverted portion of the pygypodium) and limits the amount of coelomic fluid in regions distal to the plug. As eversion proceeds, each successive transverse array of spurs arrives at the outer tip of the

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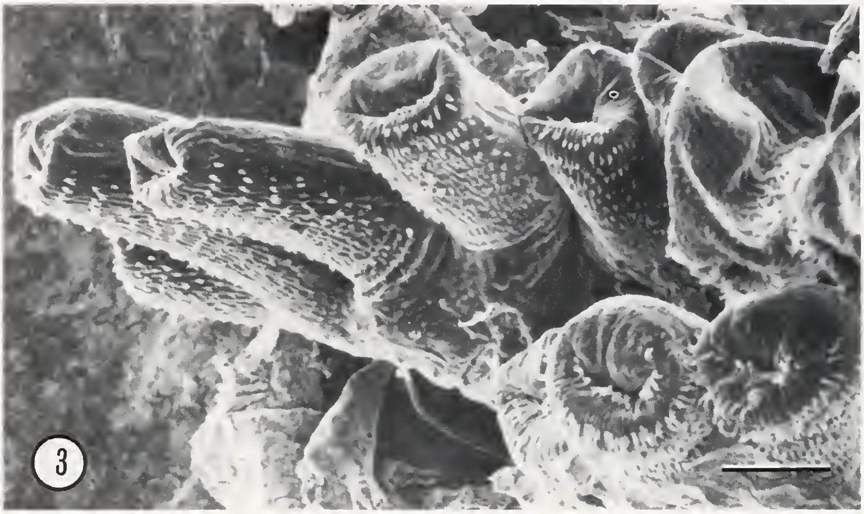
FIGURE 2a. Outline of tergum of the ninth abdominal segment (IX) and everted pygypodia, dorsal view. The pygypodial apparatus exhibits clear bilateral symmetry and all pygypodia originate from a common basal stalk which is covered by a brush of fine hairs. Only the dorsal podia are visible: the outer four on each side originate from a common branch of the main stalk, and the remaining two from a separate, more medial branch.

FIGURE 2b. Ventral view of the dorsal body wall of segments VII, VIII and IX, with the ventral pygypodia removed, showing the muscle arrangement and pleural plates (Pl). Note the abrupt change in the arrangement of the medial dorsal longitudinal muscles (*) from a parallel to a twisted one and back again, as they approach the sites of insertion on the pygypodia. The lateral dorsal longitudinal muscles (**) follow the more usual intersegmental course and are not connected to the pygypodia.

FIGURE 2c. As in Figure 2b, but with the ventral pygypodia still present. The muscles inserting on the ventral pygypodia (arrows) are probably those identified by Snodgrass (1935) as the paradorsal muscles. Some of the dorsal longitudinal muscles (*, **) seen in Figure 2b are represented here for reference.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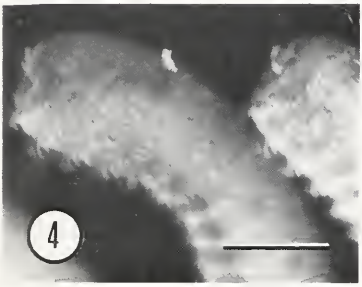
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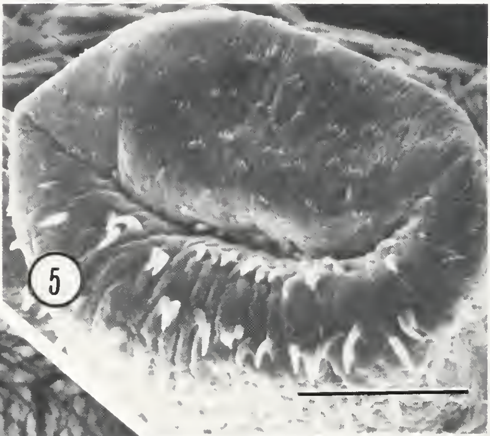
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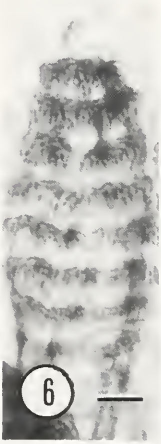
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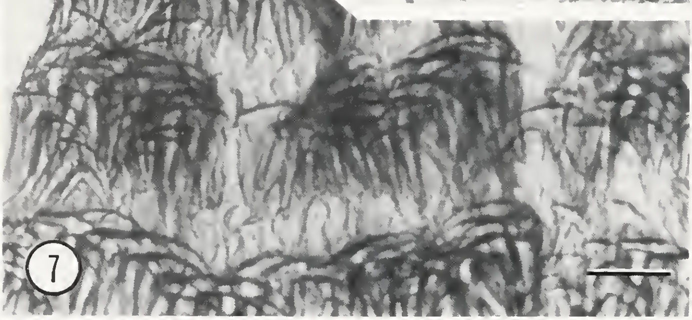
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FIGURE 3. Scanning electron micrograph of part of the pygypodial array of a *Photuris* larva, from the postero-ventral aspect, showing several partially everted dorsal and two ventral pygypodia. Note the uniformity of the transverse rows of ventral spurs on each of the podia. Bar = 0.1 mm.

FIGURE 4. Light micrograph of the tips of two fully everted pygypodia. The terminal protrusion or bulb is visible on each, and some of the rows of ventral spurs may be seen along the sides. Bar = 0.1 mm.

FIGURE 5. Ventral pygypodium from the group in Figure 3, rotated to show a view directly "into" the partially everted pygypodium. Spurs which are completely external are erect in position, while those still within the inverted portion of the podium show the flattened, packed orientation they maintain inside.

unfolding pygypodium (Fig. 5) and each individual spur springs erect and perpendicular to the integument.

Depending on the nature of the substrate, a spur may either penetrate or, if the substrate is impenetrable, tip sideways, lying nearly flat and parallel to the integument. This effect is most readily observed in spurs which are not members of recently everted arrays.

Walking. As an ancillary appendage for walking, the basal stalk of the inverted pygypodium may be used like a suction cup. The area is always damp, and the presence of even a small amount of fluid results in adhesion to the substrate. The animal may also be held by surface molecular forces in the liquid film (Wigglesworth, 1972). Secretions, possibly mucus, from the terminal abdominal segments apparently supply necessary fluid, and the whole area is rich in tufts of bristles (Figs. 2a, b) which help hold and distribute the moisture. Once attachment is made to the substrate, the larva can use its abdominal musculature to move the body forward.

Other functions. Under special circumstances, *i.e.*, when the animal is trying to right itself or is exploring unusual territory (such as a glass slide), selected pygypodia partially invert, and their tips act as suction cups (Fig. 5); the ventral rings of spurs are turned upwards out of the way, and the center of the tip forms a depression by contraction of the internal muscle fibers. Release from the suction is accomplished by everting the pygypodium and having the bulb at the tip break the seal.

DISCUSSION

Inversion or eversion of the pygypodia may be described as the result of antagonism between the podal muscles and those muscle groups which control the coelomic (or, more precisely, pseudocoelomic) pressure in the abdomen. The complex arrangement of the pygypodial muscles (and their presumably equally complex innervation) may be responsible for the high level of control exhibited during movement of individual pygypodia and of the cluster as a whole. The apparent difference between the dorsal longitudinal muscles of segment VII and the podal retractor muscles of segment VIII probably represents a modification of the typical arrangement of the larval abdominal musculature in this region.

In cross section, the pygypodial integument consists of an outer supporting ring of exo- and endo-cuticle encasing a flexible inner layer of epidermal cells and basement membrane. The cuticle, and in particular the ventral arrays of spurs, supports the cylindrical shape of the pygypodia. Totally inverted podia are compressed, yet they still remain semicylindrical. The series of transverse rings generated by the packing of the spurs supports and maintains the cylindrical shape of the inverted parts of a pygypodium, more or less in the fashion of the cartilage rings in the vertebrate trachea.

Within the endocuticle, the continuous sheet of parallel microfibrils forms a tough, elastic inner sheath, which strengthens the whole pygypodium. Thus, during eversion, the increased fluid pressure is evenly distributed throughout the hollow of the tube. There appear to be no irregularities or bulges in the integument, even when it is

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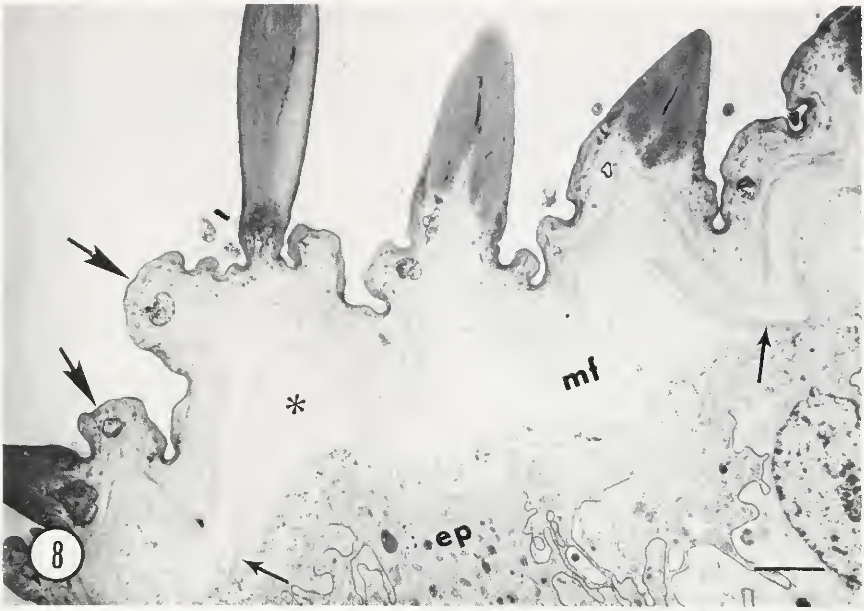
The small barb-like structures on the dorsal portion of the unfolding pygypodium are typically found on the dorsal regions of the pygypodia and are probably homologous to the ventral spurs. Bar = 0.05 mm.

FIGURE 6. Light micrograph of a fully inverted pygypodium which has been treated with potassium hydroxide to remove all cellular constituents. The compact packing of the internalized spurs is visible here. Bar = 0.03 mm.

FIGURE 7. As in Figure 6, but higher magnification. The compact "criss-cross" arrangement of the internalized spurs is clearly visible. Bar = 0.02 mm.



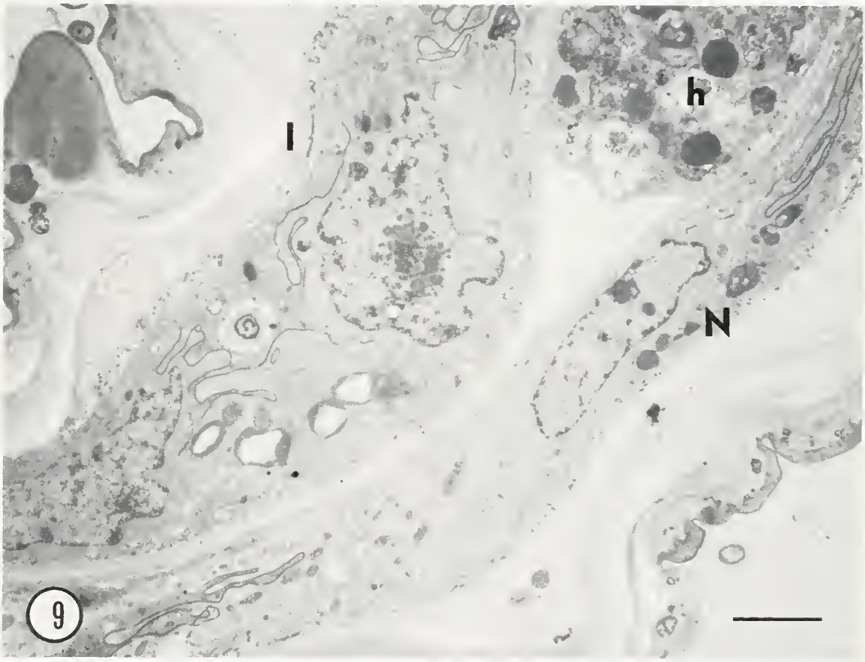
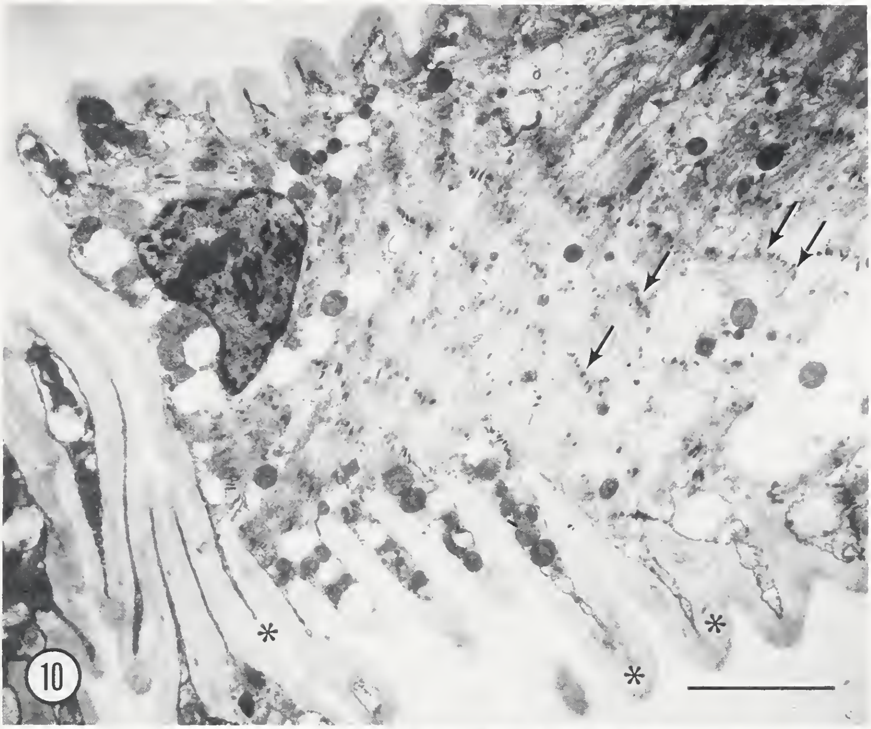


FIGURE 8. Transmission electron micrograph of transverse section through the pygypodial integument. The electron-dense spurs have broad outcroppings or folds of cuticle at their bases (large arrows) and the endocuticle shows the peak-and-valley patterning of the microfibril sheet (mf). Note the apparent attachment of the latter to the epidermis (ep; small arrow) as well as the extension of the fibrils into the spines. This view also shows the amorphous endocuticle (*). Bar = 2 μm.

FIGURE 9. TEM of part of the inverted (I) and non-inverted (N) walls of a partially retracted pygypodium. The basement membranes between the two integuments are closely apposed into a relatively tight seal which limits the space available for coelomic fluid between them. (The seal extends to and includes a hemocyte (h) which has apparently gotten caught in the gap.) The epithelial cells are irregular in shape, loosely organized, and have few sites of attachment to the basement membrane or to the cuticle. Bar = 2 μm.



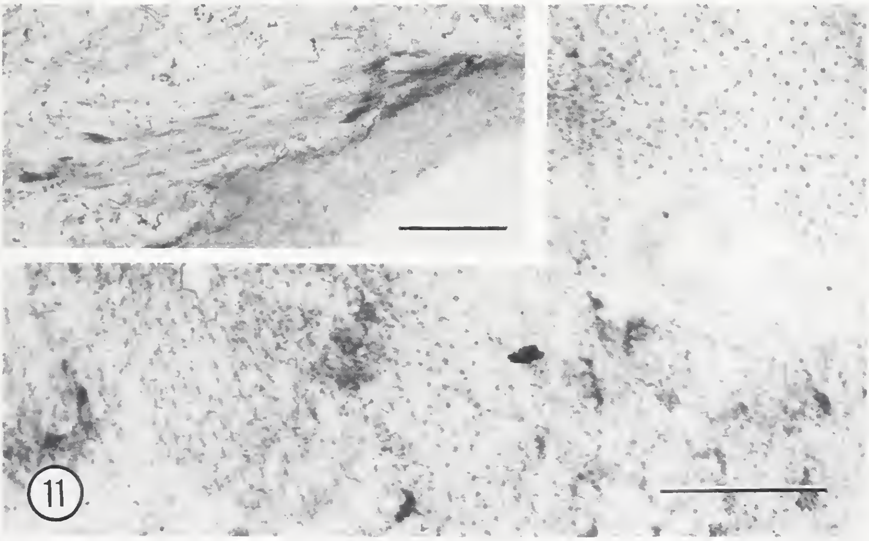


FIGURE 10. TEM of a longitudinal section of a contracted pygypodial muscle, showing the pleated appearance of the connective tissue sheath (*) and the variability in the arrangement of the Z-line material (small arrows). Bar = 0.33 μm.

FIGURE 11. TEM of a transverse section through part of a pygypodial muscle fiber. Only a small portion (upper right) of the total area shown presents the typical cross-sectional actin-myosin filament patterning because these muscles have a more or less felt-like arrangement, with parts of the fibrils going in different directions. Bar = 0.5 μm. *Inset.* Site of attachment of Z-line material to the plasma membrane. Bar = 0.5 μm.

under pressure. If the animal's abdomen is pressed with forceps, fluid leaks from the intersegmental membranes long before the pygypodia are ruptured, which suggests that the integument of these appendages is built to withstand the pressure and stress that must be generated in any hydrostatic system.

The loose folds of the exocuticle and of the microfibril sheet of the endocuticle allow these layers the necessary "give" to accommodate the changes in circumference and shape that accompany pygypodial movement. The apparently unstructured inner layers of the cuticle have no such folds, and so they must either stretch (Filshie, 1982) or flow (Vincent, 1981) as the cuticle is stressed. The connective tissue sheaths of the muscles can apparently accommodate substantial changes in muscle length, and the loose organization and highly folded or involuted surfaces of the epidermal cells are characteristic of epithelia that must withstand changes in shape (Vincent, 1981). The sparse but regular attachments of the cells to the microfibril sheet apically, and to the basement membrane proximally, must limit the displacement of the various components when the system is stressed.

The basement membrane may also play a role in generating and maintaining the tight occlusive seal between the inner and the outer walls of the plug region of a partially everted pygypodium. The basement membranes of the inverted and everted sections conform closely to one another, and so ensure that coelomic pressure will be applied to the plug (the inverted portion of the pygypodium) rather than be dissipated by the leakage of fluid between the outer and inner walls. The loose organization of the epidermal cells and the large intercellular spaces may also allow the tissues to "give" where necessary, and at the same time provide cushioning between the layers.

During eversion and inversion, the presentation or folding of the spurs may depend on the elasticity of the endocuticular microfibril sheet which interconnects all the spurs at their bases. Once released from the confines of the outer sleeve of the pygypodium, the individual spurs of each transverse array reorient from their "packed" positions to erect ones. The interconnection of the spurs by the microfibril sheet may smooth and integrate this transition so that the spurs are presented in an orderly and consistent fashion.

During inversion, the integument and associated arrays of spurs are internalized by the contraction of the pygypodial muscles. The dorsal longitudinal crease serves as a guide for the proper inversion of the integument and folding and compacting of the spurs, possibly by altering the direction of stress in the cuticle. With the seal lost around the plug, coelomic fluid may enter the pygypodium and aid in compacting the already inverted portions.

Even though the spurs lie flat when a pygypodium is in contact with smooth and impenetrable surfaces, they automatically revert back to erect positions when it is removed from these surfaces. Each spur is apparently braced, and its base reinforced by the associated portions of the underlying microfibril sheet. The design of the sheet may concentrate coelomic fluid pressure on the bases of the spurs, an arrangement which maintains undisturbed spurs in the perpendicular position, while allowing enough flexibility for spurs under external pressure to "give" or reorient.

The pygypodia may have evolved as modifications of the intersegmental membranes of the last abdominal segment. The typical larval intersegmental structure consists of antecostal cuticular folds (Snodgrass, 1935). To arrive at something like the pygypodia, one need only postulate extension of one such fold into deep and distinct pouches. The original dorsal longitudinal muscle insertions are carried in with the pouches to become associated with what are now the tips of the inverted pygypodia. Correspondingly, the ventral pygypodia may have developed in a similar manner. The observed anomalies, *i.e.*, the twisting of the dorsal musculature and the crossing over of some of the ventral muscles to the opposite side of the midline, remain unresolved puzzles requiring further work and thought.

ACKNOWLEDGMENTS

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THE FUNCTIONS OF NEMATOCYSTS IN PREY CAPTURE BY EPIPELAGIC SIPHONOPHORES (COELENTERATA, HYDROZOA)[1]

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ABSTRACT

The nematocysts of 24 siphonophore species were examined by light and scanning electron microscopy (SEM) for differences that could relate to differences in the sizes and types of prey captured. The siphonophore species in the suborder Calycophorae had 4–30 microbasic mastigophores (0.7–18.0 μl volume), and 50–2000 smaller homotrichous anisorhizas in uncoiled nematocyst batteries. The physonect siphonophore species had 4–120 stenoteles or microbasic mastigophores (1.8–40.7 μl volume), and 150–20,500 smaller homotrichous anisorhizas in coiled nematocyst batteries. The sizes of crustacean prey (primarily copepods) captured by species in both suborders increased with increasing nematocyst size and numbers. Examination by SEM of captured, but uningested prey showed that the heavily-spined threads of these nematocysts adhered to the prey surface, and primarily entangled the prey. In contrast, the tentacles of siphonophores in the suborder Cystonectae, which includes *Physalia physalis,* have only isorhizas of 1.0–18.0 μl volume with and without small spines on the threads. These nematocysts penetrate the soft-bodied prey (mostly fish larvae) of these siphonophores, but apparently do not penetrate or entangle hard-bodied prey. Thus prey capture by siphonophores differs with the sizes, numbers, and types of nematocysts present in each species. The possible functions of nematocyst batteries in tentacle spreading, and luring of large zooplankton prey are discussed.

INTRODUCTION

Siphonophore species differ in the sizes and types of prey they capture (Purcell, 1980, 1981c). This is especially intriguing because these gelatinous zooplankters appear to be passive feeders, drifting in the water with their tentacles spread (Fig. 1), and depending on prey to swim into contact with them. Dietary trends exist among the three siphonophore suborders: most calycophorans feed primarily upon small copepods; most physonects consume larger zooplankton, including copepods and other crustaceans; and cystonects consume soft-bodied prey, primarily larval fish (Purcell, 1980, 1981a, c, submitted). These dietary trends are related to behavioral and morphological characteristics of the siphonophores (Purcell, 1980). Species which consume small copepods generally swim rapidly in an arc or spiral to spread their closely-spaced tentacles, and have numerous, small gastrozooids ("stomachs") (Fig.1). In contrast, siphonophores that consume large zooplankton generally do not swim rapidly to spread their more widely-spaced tentacles, and have fewer, large gastrozooids.

The structures directly responsible for prey capture are the nematocysts in the tentacles. Calycophoran and physonect nematocysts are concentrated in nematocyst

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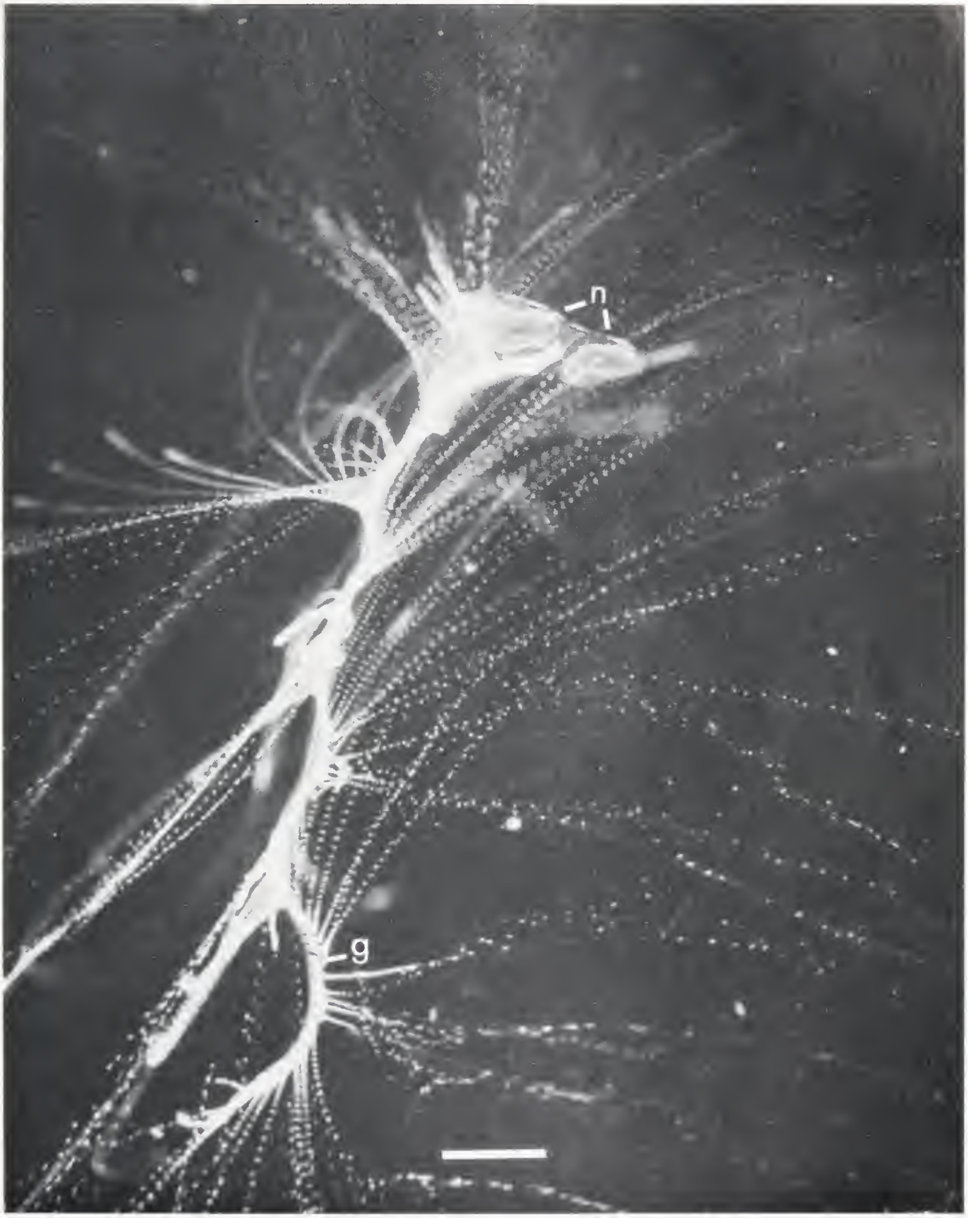


FIGURE 1. *Sulculeolaria quadrivalvis* (Suborder Calycophorae) in feeding posture with tentacles spread. The tentacles appear as a string of dots, which are the nematocyst batteries. n = Nectophores (swimming bells), g = Chain of gastrozooids (stomachs). Scale = 2 cm. Photograph by J. M. King.

batteries, one battery on each of several side branches (tentilla) of the tentacles. Nematocyst batteries consist of a cnidoband with many nematocysts, several larger nematocysts along the cnidoband, an elastic ligament, and 1 or 2 terminal filaments. The battery nematocysts fire as a unit when the terminal filaments are pulled and stretch the elastic ligament. Cystonect nematocysts are not found in these complex batteries, but may be concentrated in clumps or bands in the tentacles.

Nematocysts are classified according to the structure of the thread, which is coiled within the nematocyst capsule and everts upon discharge (reviewed in Mariscal, 1974). Historical works on siphonophore nematocysts have dealt primarily with systematic classification and the processes of nematocyst formation and discharge (Chun, 1891, 1892; Iwanzoff, 1896; Schneider, 1899, 1900; Weill, 1934; Russell, 1938, 1939; Werner, 1965). More recently, siphonophore nematocysts have been used to elucidate the process of nematocyst development (Carré, 1972, 1974a, b, c; Carré and Carré, 1973; Skaer, 1973).

Different functions have been ascribed to some of the various types of coelenterate nematocysts based on the structure of the thread, or on correlation of certain nematocyst types with specific functions. For example, an anacrophore is called a glutinant because the thread is closed at the tip while other types are presumed to be penetrants because the thread is open at the tip (Mariscal, 1974); holotrichous isorhizas in the acroraghi and catch tentacles of sea anemones presumably penetrate the victims of inter- and intraspecific agonistic interactions (Francis, 1973; Purcell, 1977). The discharge of nematocysts on prey organisms has been investigated only in *Hydra;* Toppe (1909), Ewer (1947), and Tardent and Holstein (1982) found that prey were penetrated by stenoteles and were entangled by desmonemes. Observations in Schwartz *et al.* (1983) suggest that a particular cladoceran species, which was not captured often by *Hydra* spp., might not be penetrated by the nematocysts of *Hydra,* might not stimulate the nematocysts to fire, or might be immune to the toxins. The present paper summarizes the characteristics of the nematocyst batteries and nematocysts of the three siphonophore suborders, and examines differences in the nematocysts that may mediate the differential prey capture seen in several siphonophore species.

MATERIALS AND METHODS

Prey size analysis

Siphonophores were collected in jars by SCUBA divers at 5–25 m depth primarily in the Gulf of California, the Gulf Stream, and the Sargasso Sea. Details of the methods, and of the locations and dates for collection of most siphonophores used in analysis of prey size are given in Purcell (1981c). Additional dietary data came from specimens collected during August, 1981 in the North Atlantic along the 25°N parallel, and during February and May, 1982 in the Sargasso Sea (approximately 30°77–78′W and 34°N 73°W, respectively), and in the Gulf Stream (approximately 32°N 78°W to 36°N 72°W and 33°N 77°W to 36°N 73°W). The cephalothorax length of copepods was measured using an optical micrometer at 25–400× magnification.

Microscopy

Siphonophores used for scanning electron microscopy (SEM) were collected primarily in the North Atlantic during August, 1981, as above. When the siphonophores had assumed a feeding posture with tentacles spread in 4 l clear plastic containers aboard ship, active copepods, chaetognaths, or fish larvae from plankton tows were released individually near the tentacles by pipette. The captured prey were retrieved with forceps after the prey swam into contact with the siphonophore tentacles, but before they could be ingested. Specimens were placed in 2% gluteraldehyde in sea water for 1 h, postfixed in 1% OsO_4 in sea water for 1 h, transferred at 10 min intervals into 30%, 50%, and 70% ethanol and stored at 4°C. Ethanol dehydration was followed by critical-point drying with CO_2 or Freon in a Tousimas sam-dry-780 or a Bowmar SPC-900 critical-point drying chamber. Specimens were mounted on stubs, rotary-coated with gold in a Denton DSM-5 or an International Scientific Instruments (ISI) PS-2 vacuum evaporator, and examined in a JEOLCO V3 or an ISI Alpha-9 scanning electron microscope in order to examine the effect of nematocysts on prey. Small pieces of Nitex screen were swept through extended tentacles and treated as above, in order to examine the thread structure of discharged nematocysts. One or 2 copepods, and 1 screen were examined for each siphonophore species.

Siphonophores used for light microscopy first were relaxed in $MgCl_2$ in sea water until the tentacles no longer contracted when touched, and then preserved in 5% formalin. The length, width, and depth of nematocyst batteries were measured at 40–400× magnification on a compound microscope with an optical micrometer. The maximum length and width of undischarged nematocysts from disrupted batteries were measured at 400–1000×. The numbers of anisorhizas (haplonemes) were approximated from the surface area (length × width) of the cnidoband divided by the maximum cross-sectional area of the anisorhizas; the depth of the cnidoband corresponds to their length. The large microbasic mastigophores and stenoteles (heteronemes) were counted, and their volumes were estimated according to the formula for the volume of an elipsoid, $4ab^2/3$, where $a = \text{length}/2$, and $b = \text{diameter}/2$. The dimensions of at least 5 nematocysts of each type were measured for each specimen, and 2–6 specimens were examined in each species. The measurements are from one specimen, or are maximum and minimum values where substantial differences existed among several specimens within a species.

Terminology

Nematocyst classification and terminology used herein is as presented by Mariscal (1974) based on the structure of the nematocyst thread (Fig. 2). Simply, haplonemes are nematocysts without a well-defined shaft at the base of the thread; isorhizas have threads of equal diameter throughout, while the base of the thread of an anisorhiza is slightly dilated; holotrichous indicates that spines occur along the length of the thread, and atrichous threads have no spines. Heteronemes have a well-defined shaft; microbasic mastigophores have a short shaft of equal diameter throughout; stenoteles have a short shaft of unequal diameter, with 3 well-developed spines. Desmonemes, acrophores, and anacrophores have threads with closed tips and no spines.

RESULTS

Measurements and photographs of nematocyst batteries and nematocysts are presented for the 3 siphonophore suborders: Calycophorae (Table I, Fig. 3), Physonectae (Table II, Fig. 4), and Cystonectae (Table III, Fig. 5). Each table and figure has been organized by siphonophore family, and arranged in order of increasing nematocyst and nematocyst battery sizes, as far as was possible. Differences in corresponding nematocyst dimensions in the tables and figures may be due to effects of preservation, and to a 20% reduction in length and diameter in capsules of discharged nematocysts (Tardent and Holstein, 1982). The batteries and nematocysts were similar enough among species of each family that only representative illustrations are given for each family in Figures 3, 4, and 5.

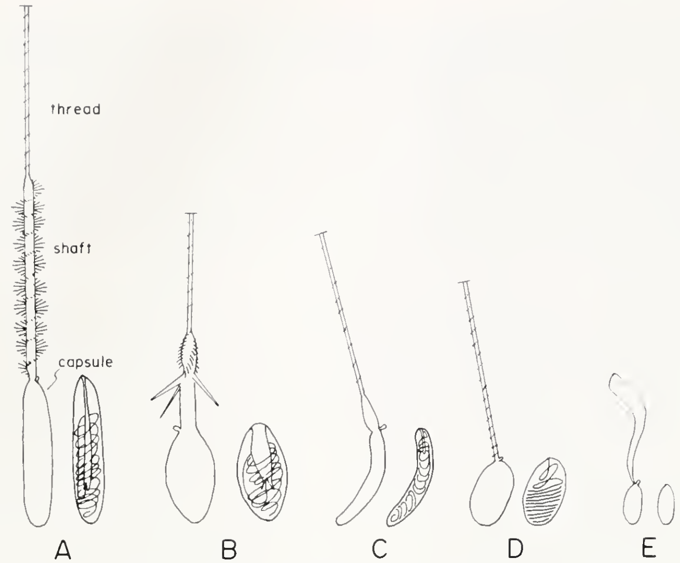


FIGURE 2. Discharged and undischarged nematocysts from siphonophore tentacles. A. Microbasic mastigophores (heteronemes). B. Stenoteles (heteronemes). C. Holotrichous anisorhizas (haplonemes). D. Holotrichous isorhizas (haplonemes). E. Anacrophore (rhopalonemes). After Iwanzoff (1896).

Suborders Calycophorae and Physonectae

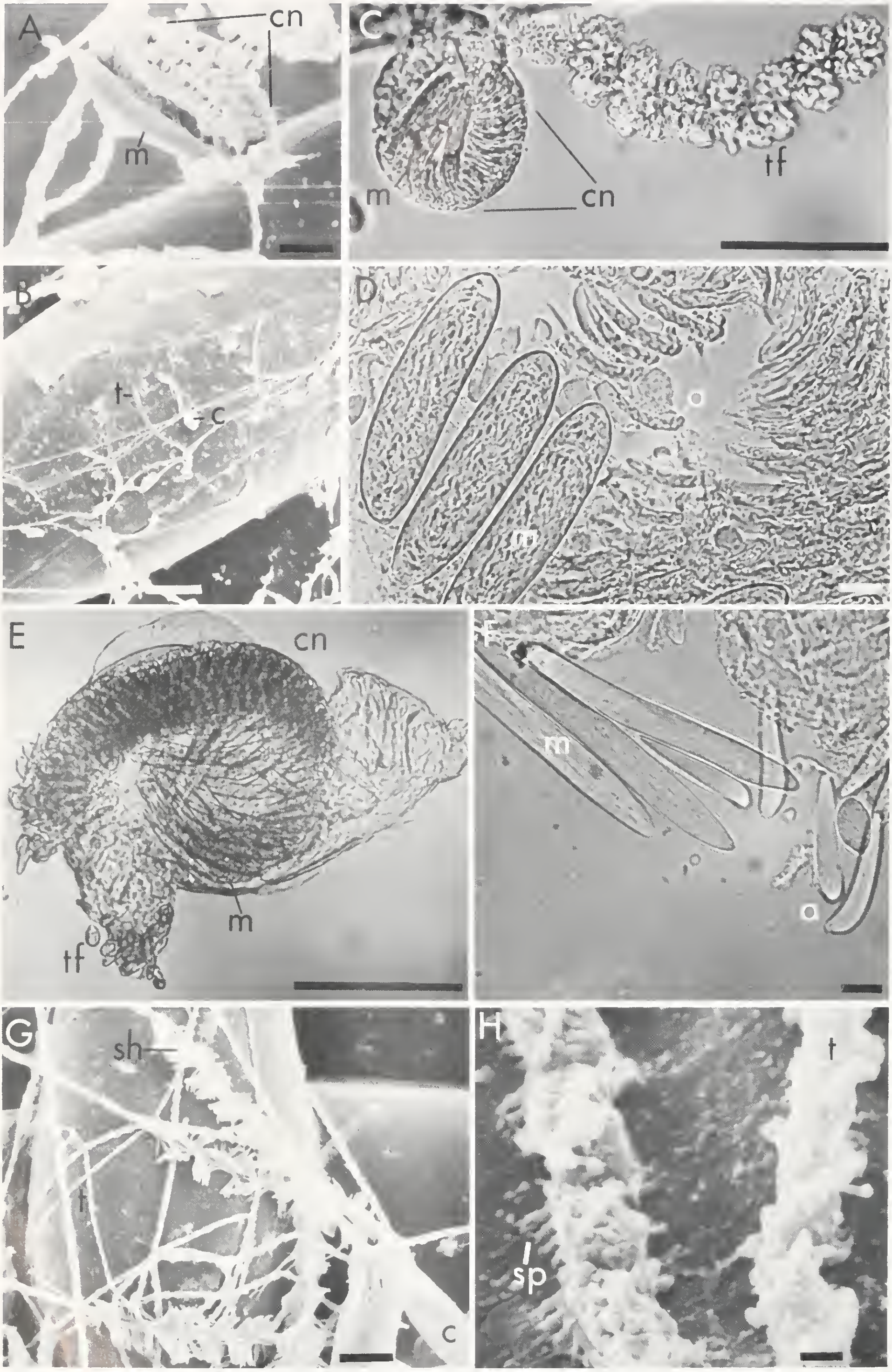
Each of the species examined in these suborders had nematocyst batteries that contained numerous haplonemes of one type in the cnidoband and several larger heteronemes of one type, and 1 or 2 types of small nematocysts in the terminal filaments. The nematocyst battery structure was consistent within each suborder, but sizes of nematocysts differed among species (Tables I and II). The ranges of nematocyst sizes within species in Tables I and II result from the fact that small specimens within a species had fewer and somewhat smaller heteronemes than large specimens. This fact may explain differences in measurements made by other authors, as follow. An increase in the cnidoband length with siphonophore size was also noticeable in *Stephanophyes superba, Athorybia rosacea, Forskalia edwardsi,* and *Agalma* spp., where specimens spanning a large size range were available. Large specimens were used in calculations of the numbers of anisorhizas (haplonemes, Tables I and II) and volumes of the heteronemes (Table IV).

The nematocyst batteries of the calycophoran species within the families Sphaeronectidae, Diphyidae, and Abylidae were alike, having a long, straight cnidoband (Fig. 4A, I, L); those of the families Hippopodiidae and Prayidae were more rounded (Fig. 4C, E). The holotrichous anisorhizas (haplonemes) (Fig. 3D, F, N) of the uncoiled cnidoband of calycophorans were classified by Weill (1934) in *Eudoxoides (= Diphyes) spiralis* (Bigelow, 1911) (22 × 6–32 × 7 μm), *Hippopodius hippopus (= neapolitanus)* (30 × 5 μm), *Rosacea (= Praya) cymbiformis* [60 × 6.5 μm, data from Iwanzoff (1896), where identification of the specimen was uncertain; those measurements and those for the heteronemes (below) differ greatly from those on *R. cymbiformis* in Table I], and *Abylopsis tetragona* (62 × 10 μm). Russell (1939) described the haplonemes of *Muggiaea atlantica* as atrichous anisorhizas (13–18 × 3–4 μm). The elongate heteronemes were microbasic mastigophores (Fig. 3A, C, D, E, F, G, I), as classified by Weill (1934) for *E. spiralis* (62 × 9–85 × 11 μm), *H. hippopus* (90 × 17–

TABLE I

Battery nematocysts of siphonophores in the suborder Calycophorae

		Cnidoband				
		Heteronemes		Haplonemes		
	Battery dimensions (μm)	(μm)	(No.)	(μm)	(No.)	Terminal filament nematocysts (μm)
Family Sphaeronectidae						
Sphaeronectes gracilis (Claus, 1873)	48 × 26 × 18	36.0 × 7.0	4	12.0 × 2.0 7.0 × 5.0	50	4.0 × 3.0 3.0 × 1.5
Family Hippododiidae						
Hippopodius hippopus (Forskal, 1776)	96 × 96 × 46	78.0–84.0 × 17.0	7–10	26.0 × 5.5	200	6.0 × 6.0
Family Prayidae						
Rosacea cymbiformis (Chiaje, 1822)	255 × 120 × 50	67.0 × 8.0	25–30	32.0 × 6.0 11.0 × 7.0	400	9.6 × 4.4 8.0 × 8.0
Stephanophyes superba (Chun, 1888)	1500 × 60 × 84	111.0 × 13.0	32–50	45.0 × 9.0 23.0 × 13.0	2000	27.0 × 9.0 18.0 × 10.0
Family Diphyidae						
Muggiaea atlantica (Cunningham, 1892)	120 × 52 × 30	36.0 × 6.0	6	15.0 × 4.0 9.0 × 6.0	300	6.0 × 4.0 6.0 × 2.0
Chelophyes appendiculata (Eschscholtz, 1829)	150 × 52 × 36	66.0 × 6.0	6	15.5 × 4.0 12.0 × 7.0	450	10.0 × 6.0 9.0 × 3.0
Sulculeolaria quadrivalvis (Blainville, 1934)	114 × 66 × 30	47.5 × 7.5	8	20.0 × 5.0 12.5 × 9.5	200	7.5 × 2.5 7.5 × 7.5
S. monoica (Chun, 1888)	168 × 66 × 30	60.0 × 10.0	8	22.5 × 5.5 12.5 × 10.0	—	12.5 × 5.0
Diphyes dispar (Chamisso and Eysenhardt, 1821)	270 × 78 × 42	97.5 × 11.0	12	15.0 × 7.5 20.0 × 12.0	250	15.0 × 9.0
Family Abylidae						
Bassia bassensis (Quoy and Gaimard, 1833)	315 × 100 × 70	75.5 × 14.0	8	36.0 × 8.5 14.0 × 9.0	400	11.0 × 8.0 12.0 × 4.0
Abylopsis tetragona (Quoy and Gaimard, 1827)	891 × 198 × 70	145.0–155.0 × 15.0	20–21	52.5 × 10.0 25.0 × 12.5	800	23.0 × 3.0 15.0 × 5.0
Abyla trigona (Quoy and Gaimard, 1827)	396 × 248 × 120	153.0 × 15.0	11–13	54.0 × 12.5 23.0 × 12	400	15.0 × 5.0



The following abbreviations are used in Figures 3–5: a = anisorhiza, c = nematocyst capsule, cn = cnidoband, el = elastic ligament, i = isorhiza, m = microbasic mastigophore, t = nematocyst thread, tf = terminal filament, s = stenotele, sh = nematocyst shaft, and sp = spines on nematocyst thread.

FIGURE 3. Suborder Calycophorae. **A.** Nematocyst battery of *Sphaeronectes gracilis* (Fam. Sphaeronectidae), 1000×, scale = 10 μm. **B.** Nematocysts (anacrophores) from the terminal filaments of *S.*

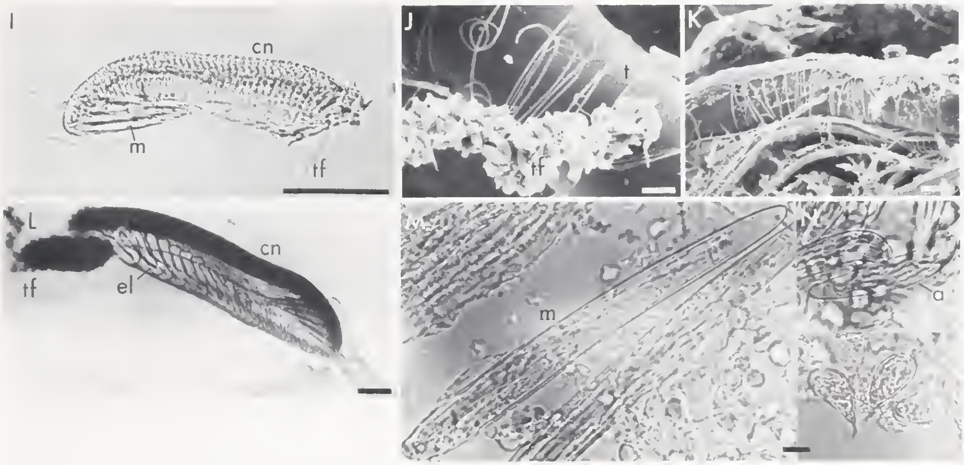


FIGURE 3 (con't). I. Nematocyst battery of *Diphyes dispar* (Fam. Diphyidae), 160×, scale = 100 μm. J. Terminal filament of *Sulculeolaria quadrivalvis* (Fam. Diphyidae), 1000×, scale = 10 μm. K. Copepod antenna wrapped by the nematocyst threads of *S. quadrivalvis*, 400×, scale = 25 μm. L. Nematocyst battery of *Abylopsis tetragona* (Fam. Abylidae), 50×, scale = 100 μm. M, N, O. Nematocysts of *Abyla trigona:* M. Large heteronemes, N. Haplonemes (anisorhizas) from the cnidoband, O. Haplonemes (isorhizas) from the battery tip. 400× scale = 10 μm.

95 × 18 μm), *R. cymbiformis* (250 × 16 μm), and *A. tetragona* (195 × 17 μm); and by Russell (1938) for *M. atlantica* (36–43 × 6–7.5 μm), with prominent spines on the shaft (Fig. 3G) and smaller spines along the remainder of the thread (Fig. 3H). Heteronemes of *Stephanophyes superba* and *Diphyes dispar (= Dormasia picta)* were measured at 120 μm and 100 μm in length by Chun (1891) and (1892), respectively. At the free end of the batteries were several smaller rounded nematocysts (3E, I, L, O), probably holotrichous isorhizas (haplonemes, as pictured in Iwanzoff, 1896), with long cnidocils, the ciliary sensory receptors which trigger nematocyst discharge (*e.g.*, Carré and Carré, 1980). The single terminal filament on each battery contained nematocysts with club-shaped threads; anacrophores (Fig. 3B), as identified by Weill (1934) in *E. spiralis* (5–6 × 2 μm), *H. hippopus* (12 μm), *R. cymbiformis* (22 × 8 μm), and *A. tetragona* (22 × 8 μm), and by Russell (1938) in *M. atlantica* (5–6 × 2 μm), and/or desmonemes as identified by Weill (1934) in *E. spiralis* (7–10 × 4–6 μm), *H. hippopus* (7 × 7.5 μm), and *A. tetragona* (30 × 13 μm), and by Russell (1938) in *M. atlantica* (7–10 × 4–6 μm). Some measurements of nematocysts in the batteries of other calycophoran species exist: (Family Diphyidae) *Dimophyes arctica* (Chun, 1897), heteronemes 280 μm, *Lensia conoidea* (Kefferstein and Ehlers, 1860), heteronemes ~ 53 × 7 μm; *Sulculeolaria biloba* (Sars, 1846), heteronemes = 56–58 × 7–8 μm, haplonemes = 19–24 × 3–5 μm and 9–11 × 7–8 μm (in Totton, 1965); (Family Abylidae) *Enneagonium hyalinum* (Quoy and Gaimard, 1827), heteronemes = 80 μm long, haplonemes = 40 μm long (in Chun, 1892). The terminal filaments of *Sulculeolaria* spp., especially *S. quadrivalvis*, were very adhesive, and had haplonemes with holotrichous threads (Fig. 3J).

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gracilis. 1000×, scale = 10 μm. C. Nematocyst battery of *Hippopodius hippopus* (Fam. Hippopodiidae) 160×, scale = 10 μm. D. Nematocysts of *H. Hippopus*, 400×, scale = 10 μm. E. Nematocyst battery of *Rosacea cymbiformis* (Fam. Prayidae), 160×, scale = 100 μm. F. Nematocysts of *R. cymbiformis*, 400×, scale = 10 μm. G. Discharged nematocysts (microbasic mastigophores) of *R. cymbiformis*, 1000×, scale = 10 μm. H. Nematocyst threads of *R. cymbiformis* adhering to the surface of a chaetognath, 8000×, scale = 1 μm.

TABLE II

Battery nematocysts of siphonophores in the suborder Physonectae

		Cnidoband				
		Heteronemes		Haplonemes		
	Battery dimensions (μm)	(μm)	(No.)	(μm)	(No.)	Terminal filament nematocysts (μm)
Family Athorybiidae						
Athorybia rosacea (Forskal, 1775)	102 × 60 × 60	65.0 × 22.5	4–50	32.5 × 7.5	800	7.0 × 2.5 5.0 × 5.0
Family Forskaliidae						
Forskalia edwardsi (Kölliker, 1853)	420 × 180 × 180	38.0 × 22.5	20–30	30.0 × 5.5	3000	13.0 × 5.0
Family Agalmidae						
Cordagalma cordiformis (Totton, 1932)	50 × 32.5 × 32.5	22.5 × 12.5	4–7	22.5 × 6.0 6.0 × 6.0	150	3.0 × 4.5
Nanomia bijuga (Chiaje, 1841)	445 × 248 × 240	32.0 × 16.0	15–35	22.5 × 5.5	4500	11.0 × 4.5
Nanomia cara (A. Agassiz, 1865)	1400 × 680 × 680	62.5 × 22.5	70–80	72.5 × 12.5	14,000	17.5 × 10.0 12.5 × 10.0
Agalma elegans (Sars, 1846)	396 × 120 × 120	130.0 × 20.0–180.0 × 28.0	11–30	35.0 × 5.0	17,000	5.0 × 5.0–7.5 × 7.5
Agalma okeni (Eschscholtz, 1825)	1164 × 680 × 680	112.5 × 20.0–135.0 × 24.0	30–120	47.5 × 6.0	20,500	7.0–7.0
Family Physophoridae						
Physophora hydrostatica (Forskal, 1775)	1260 × 390 × 390	109.0 × 29.0	—	32.5 × 6.2	—	—

The nematocyst batteries of physonect siphonophores had a spirally coiled cnidoband (Fig. 4A, D, K), with the exceptions of *Cordagalma cordiformis* in which it is short and uncoiled (Fig. 4G, H), and *Physophora hydrostatica* in which it is loosely coiled (Fig. 4Q). The holotrichous anisorhizas (Fig. 4C, G, L, M) of the cnidoband of physonects were classified by Weill (1934) in *Forskalia* sp. (37 μm long), *Physophora hydrostatica* [30–30.5 × 7–7.5 μm (Iwanzoff, 1896)], *Agalma clausi* (Bedot, 1888) (60 μm long) and *Agalma elegans (= sarsi)/Agalma (= Agalmopsis) elegans* (22 × 12 μm), and by Russell (1939) in *A. elegans* (up to 75 × 6–7 μm), and by Carré (1968) in *Cordagalma cordiformis* (16 × 2 μm). The large, ovoid heteronemes were stenoteles in most species examined: *Athorybia rosacea* (Fig. 4B, C, as in Schneider, 1900), *Forskalia* spp. [Fig. 4E, as in Weill, 1934 (55 × 25 μm)], *Cordagalma cordiformis* [Fig. 4G, as in Carré, 1968 (17.5 × 8.5 μm)], *Nanomia bijuga* (Fig. 4L, N, probably also *N. cara*), and *Physophora hydrostatica* [Fig. 4Q, R, S, as in Schneider (1900) although Iwanzoff (1896) shows microbasic mastigophores ~ 100–150 μm long]; but were microbasic mastigophores in *Agalma elegans* [Fig. 4O, P, as in Iwanzoff, 1896 (90 × 30 μm); Weill, 1934; and Russell, 1939 (185–205 × 25–28 μm)], *A. clausi* [in Weill, 1934 (220 μm long)], and probably also in *A. okeni*. *Cordagalma cordiformis* also had several rounded isorhizas at the battery tip (as in Carré, 1968). The 1 or 2 terminal filaments per battery had 1 or 2 nematocyst types, classified in Weill (1934) as acrophores and desmonemes in *Forskalia* sp. (15 × 7.5 μm), *A. clausi* (20 μm long, and 12.5–12.5 μm), and *A. elegans* (21 × 15 μm and 10 × 10 μm), and in Russell (1938) as anacrophores and desmonemes in *A. elegans* (7 × 2–20 × 5 μm and 6–7 × 6 μm). *Halistemma rubrum* (Vogt, 1852) (Family Agalmidae) is reported to have nematocysts 60–65 × 7 μm (haplonemes) and 65–70 × 20 μm (heteronemes) in the cnidoband (Totton, 1965), although Iwanzoff (1896) comments on the enormous size of the nematocysts (1120 × 120 μm). *Apolemia uvaria* (Lesueur, 1811) (Family Apolemiidae) is an unusual physonect species in that it lacks nematocyst batteries. The nematocysts in the tentacles (birhophaloides, 24 × 15 and 15 × 9 μm) have a thread with two dilations near the base, and are unique to *A. uvaria* (as in Carré and Carré, 1973). The diet is also unusual in having a large proportion of gelatinous zooplankton (Purcell, 1981c).

The species of calcyophoran and physonect siphonophores for which dietary data are available are listed in Table IV, arranged in order of increasing mean size of copepods found in the gastrozooids. Copepods comprised 80–100% of the prey organisms in the calycophorans examined, but only 14–80% in the physonectids, which generally contained a variety of larger zooplankton [hyperiid amphipods, decapod larvae, and "shrimp" (including euphausiids)] (Purcell, 1980, 1981c). Thus, mean prey sizes based only on copepods underestimate the actual sizes of prey consumed by physonects. Even so, prey size was positively correlated with nematocyst size, expressed as the volume of the large heteronemes ($P < 0.001$, Kendall Rank Correlation, Sokal and Rohlf, 1969). Species with large heteronemes also tended to have larger haplonemes in the cnidoband (Tables I and II; $P < 0.05$, Kendall Rank Correlation). The total number of nematocysts in a battery also was significantly correlated with prey size ($P < 0.01$). Thus, as numbers and sizes of nematocysts in batteries increased, so did the size of prey captured by the various species. This trend would be stronger if prey other than copepods were considered in the analysis. The numbers of nematocyst batteries per tentacle, the numbers of tentacles per siphonophore, and the total numbers of batteries per siphonophore display a general tendency for species that capture large prey to have fewer tentacles, and fewer (but larger) batteries per tentacle (Table IV). These values span the range found in the available specimens, but should not be understood to represent the limits for any species; colonies of *Rosacea cymbiformis*

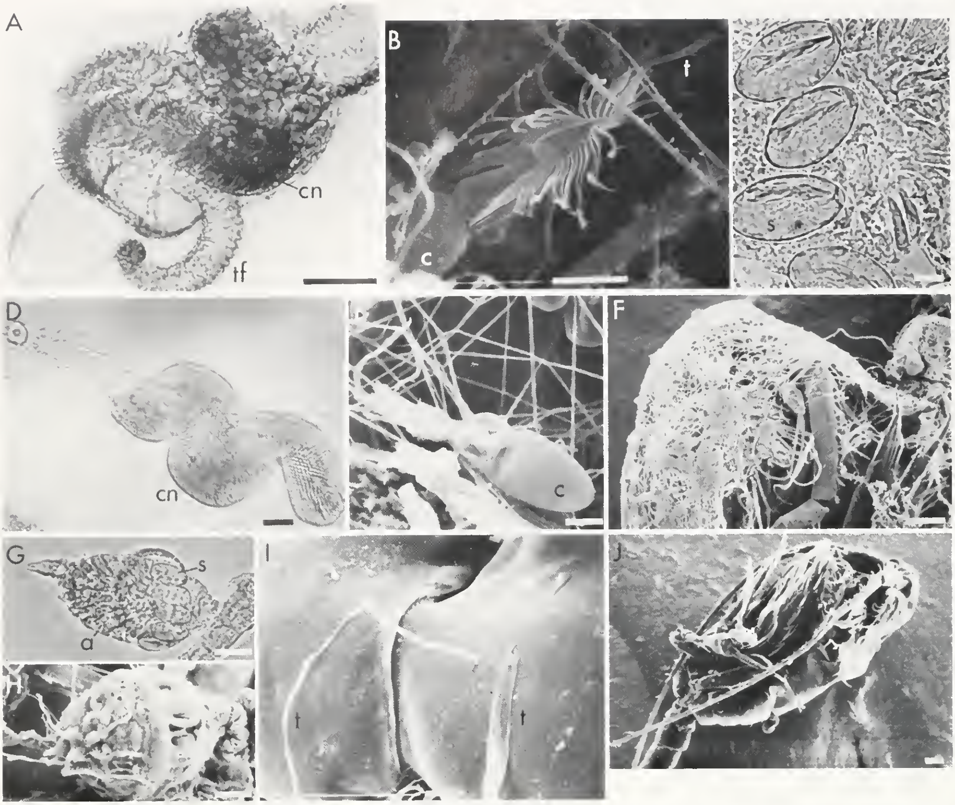

FIGURE 4. Suborder Physonectae. A. Nematocyst battery of *Athorybia rosacea* (Fam. Athorybiidae), 100×, scale = 100 μm. B. Nematocyst shaft of a stenotele from *A. rosacea,* 2000×, scale = 10 μm. C. Nematocysts of *A. rosacea,* 400×, scale = 10 μm. D. Nematocyst battery of *Forskalia edwardsi* (Fam. Forskaliidae), 40×, scale = 100 μm. E: Nematocyst (stenotele) of *F. edwardsi,* 1000×, scale = 10 μm. F. Copepod captured by *F. edwardsi,* 100×, scale = 100 μm. G. Nematocyst battery of *Cordagalma cordiformis* (Fam. Agalmidae), 200×, scale = 25 μm. H. Nematocyst battery of *C. cordiformis,* 1000×, scale = 10 μm. I. Nematocyst threads of *C. cordiformis* adhering to a copepod, 1000×, scale = 10 μm. J. Uncaptured copepod, 50×, scale = 100 μm.

and *Stephanophyes superba,* in particular, reach much greater lengths, and therefore have many more tentacles.

The effect of calycophoran and physonect nematocysts on prey was dramatic—the nematocyst threads wrapped and entangled copepod prey (Fig. 3K; 4F, M). Nematocyst threads with and without spines adhered to the surfaces of copepods, chaetognaths, and Nitex fibers (Fig. 3B, G, H, J, K; 4I, M). The spines along the length of the threads were affixed to the prey surface wherever they were in contact. The flattened threads of *Cordagalma cordiformis* nematocysts were unlike the tubular threads of other species; the tips of these unspined threads clearly adhered to crustaceans (Fig. 4I). Penetration of the exoskeleton by the nematocyst threads was not obvious in any of the copepods prepared for SEM. Threads may have entered joints in the exoskeleton in a few cases, such as when the stenoteles of *Nanomia bijuga* oriented along the cephalothorax/abdominal joint of a copepod (Fig. 4N). Nematocyst threads penetrated, as well as adhered to soft-bodied prey.

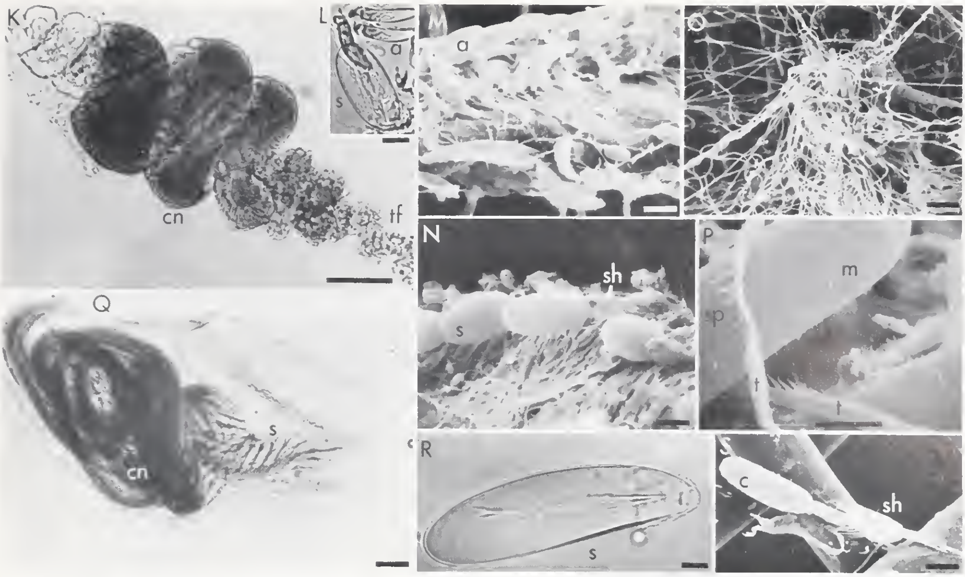

FIGURE 4. (con't). K. Nematocyst battery of *Nanomia bijuga* (Fam. Agalmidae), 100×, scale = 100 μm. L. Nematocysts of *N. bijuga*, 400×, scale = 10 μm. M. and N. Nematocysts of *N. bijuga* discharged on a copepod antenna, and along the cephalothorax/abdominal joint, 1000×, scales = 10 μm. O. Discharged nematocyst battery of *Agalma okeni* (Fam. Agalmidae), 100×, scale = 100 μm. P. Nematocyst threads of *A. okeni*, 2000×, scale = 5 μm. Q. Nematocyst battery of *Physophora hydrostatica* (Fam. Physophoridae), 50×, scale = 100 μm. R. Nematocyst of *P. hydrostatica*, 400×, scale = 10 μm. S. Discharged stenotele of *P. hydrostatica*, 400×, scale = 25 μm.

Suborder Cystonectae

The tentacles of cystonect siphonophores lack complex nematocyst batteries (Fig. 5A, E), but nematocysts occur in bands in *Physalia physalis* (Fig. 5A) and in clusters in *Rhizophysa filiformis* (Fig. 5F). All nematocysts in cystonect tentacles are haplonemes (isorhizas) (Table III, Fig. 5B, G, H). Homotrichous threads with minute spines were found in *Physalia physalis* (Fig. 5C, as in Mackie, 1960; and Cormier

TABLE III

Tentacle nematocysts of siphonophores in the suborder Cystonectae

	Haplonemes (μm)
Family Physalidae	
Physalia physalis	27.5 × 27.5
(L) 1758	12.5 × 12.5
Family Rhizophysidae	
Bathyphysa sibogae	17.0 × 17.0
(Lens and van Riemsdijk, 1908)	10.0 × 10.0
Rhizophysa filiformis	32.5 × 32.5
(Forskal, 1775)	12.5 × 12.5
Rhizophysa eysenhardti	
(Gegenbaur, 1859)	20.0 × 20.0

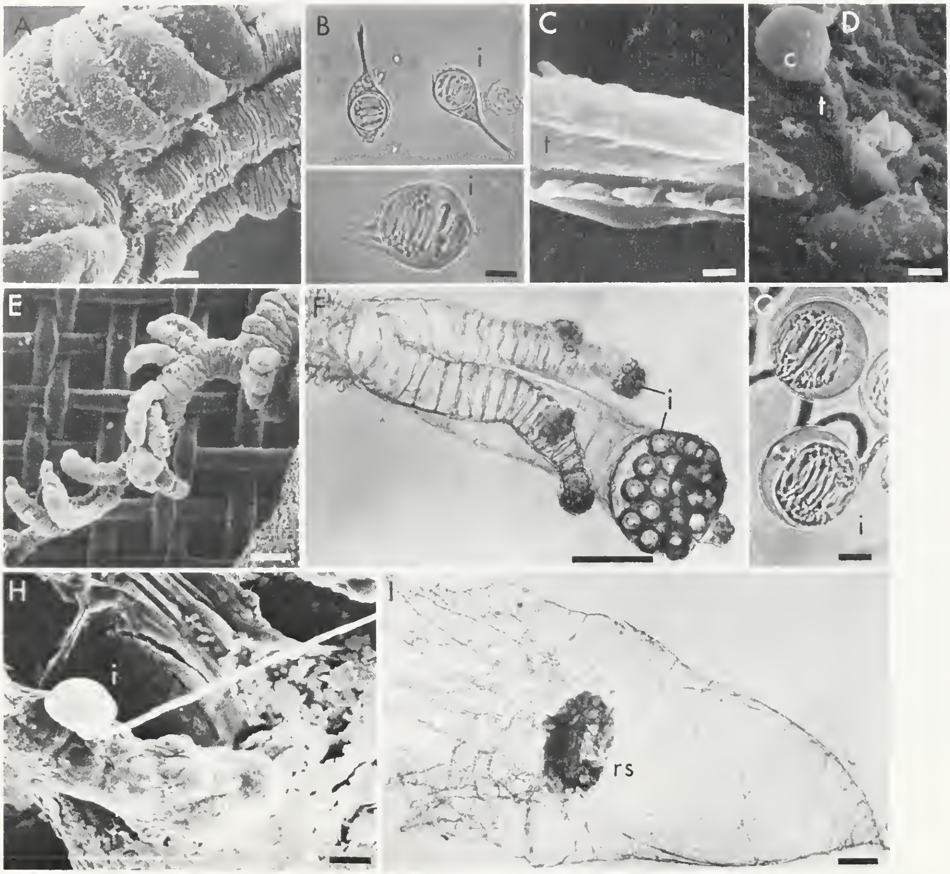

FIGURE 5. Suborder Cystonectae. A. Portion of unbranched tentacle of *Physalia physalis* (Fam. Physalidae), 50×, scale = 200 μm. B. Nematocysts of *P. physalis* in cells with fibrillar attachments to the tentacles, 400×, scale = 10 μm. C. Nematocyst thread of *P. physalis*, 8000×, scale = 1 μm. D. Nematocyst of *Rhizophysa eysenhardti* (Fam. Rhizophysidae) penetrating under the scales of a fish larva, 1000×, scale = 10 μm. E. Branched tentacle of *Rhizophysa eysenhardti*, 100×, scale = 100 μm. F. Tentacle tip of *R. filiformis*, 100×, scale = 100 μm. G. Nematocysts of *R. filiformis*, 400×, scale = 10 μm. H. Discharged nematocyst of *R. eysenhardti*, 1000×, scale = 10 μm. I. Appendage with a red spot (rs) from the tentacles of *R. filiformis*, 25×, scale = 200 μm.

and Hessinger, 1980) and *R. filiformis*, and apparently atrichous threads in *R. filiformis* and *R. eysenhardti* (Fig. 5H). The spines on the nematocyst threads of *P. physalis* are too small to be discerned with certainty by light microscopy; therefore the nematocysts were thought to be atrichous isorhizas [Weill, 1934, *Physalia arethusa (= physalis)*, 15–42 μm diameter; Totton, 1960; also measured by Lane and Dodge, 1958 (26.8 × 26.8 μm and 11.3 × 11.3 μm)].

The nematocysts of cystonects are much less diverse than those of calycophorans and physonects, and lack the conspicuous spination on the threads. The nematocysts are similar in size among cystonect species (Table III), and there is little diversity apparent in diet. *Physalia physalis* consumes fish larvae, small cephalopods, large chaetognaths, and leptocephallus larvae of eels (Purcell, submitted), as well as some larger fish to 10 cm in length (*e.g.*, Wilson, 1947); the other species consume fish larvae (Purcell, 1981a, c). Those data show that cystonect siphonophores consume

TABLE IV

Calycophoran (C) and physonect (P) siphonophores listed in order of increasing prey size (copepods only)

Species	Sub-order	Mean prey size (mm)	Large nematocyst volume (μl)*	Nematocysts per battery (no.)*	Batteries per tentacle (no.)	Tentacles (no.)	Number of batteries
Muggiaea atlantica	C	0.36	0.68	306	20	20–30	400–600
Sphaeronectes gracilis	C	0.36	0.92	54	25–30	20–60	500–1800
Chelophyes appendiculata	C	0.42	1.24	456	25–30	10–20	250–600
Cordagalma cordiformis	P	0.45	1.84	157	60–70	20–30	1200–2100
Sulculeolaria quadrivalvis	C	0.57	1.40	208	80–90	85–520	6800–46,800
Bassia bassensis	C	0.79	7.74	408	5–10	5–15	25–150
Athorybia rosacea	P	0.84	17.22	850	120	1–10	120–1200
Hippopodius hippopus	C	0.86	5.32	210	30–100	10–20	300–2000
Rosacea cymbiformis	C	0.98	2.25	430	80–90	10–100	800–9000
Diphyes dispar	C	0.99	6.46	262	40–50	90–180	3600–9000
Abyla trigona	C	1.10	18.02	413	10–20	10–20	100–400
Forskalia edwardsi	P	1.17	9.94	3030	10–20	10–320	100–6400
Nanomia bijuga	P	1.18	4.29	4535	15–20	15–20	225–400
Agalma elegans	P	1.33	27.21–73.85	17,030	12–50	5–30	60–150
Stephanophyes superba	C	1.52	9.82	2050	30–40	10–30	300–1200
Agalma okeni	P	1.97	40.69	20,620	15–35	2–9	30–315

* Calculated from values in Table I and II.

only prey lacking hard exoskeletons. Their nematocysts clearly penetrate soft-bodied prey (Fig. 5D). No discharged nematocysts penetrated or adhered to a shrimp (*Leander* sp.) that was thrust repeatedly into the tentacles of *P. physalis*. That result could be due to failure of the nematocysts to discharge or failure to affect the prey.

DISCUSSION

The large majority of the nematocysts in the battery complex of calycophorans and physonects appear to adhere to and entangle crustaceans, their principle prey. The threads of nematocysts in the terminal filaments (anacrophores and desmonemes) have closed, rounded ends. Anisorhizas, by far the most numerous nematocysts of the cnidobands, had spines along the length of the threads which adhered to all surfaces. The long, spined shaft of microbasic mastigophores also adhered to surfaces. To date, only stenoteles in *Hydra* have been shown to penetrate crustacean exoskeleton (Toppe, 1909; Ewer, 1947; Tardent and Holstein, 1982). The stenoteles of *Hydra* are forcefully ejected from the tentacle and penetrate crustacean exoskeleton presumably by the impact of the three stylets, which form a "pointed arrowhead" (Tardent and Holstein, 1982). Mariscal (1974) cited his unpublished results that showed penetration of prey by Anthozoan microbasic p-mastigophores and basitrichous isorhizas, but did not specify the prey used. Most siphonophore nematocysts do not appear to penetrate crustacean exoskeleton. Only physonect siphonophores have penetrating stenoteles, but adhering anisorhizas outnumbered stenoteles in the cnidobands of physonects by 20–40:1 (*C. cordiformis*) to ~200:1 (*Nanomia* spp.).

These results suggest that most nematocysts of calycophoran and physonect siphonophores function to entangle crustacean prey. The nematocyst threads clearly adhere to surfaces, however the mechanism of adhesion is unknown. Anthozoan nematocysts adhere to glass slides and to cleaned gastropod shells (*e.g.*, Sandberg *et al.*, 1971; Mariscal, 1972). Similarly, tentaculate ctenophores rely only upon a glue produced by colloblasts on the tentacles to adhere to, and capture, the same crustacean zooplankton prey (Franc, 1978).

The fact that the threads of most of these siphonophore nematocysts appear not to penetrate the prey raises questions about their toxicity. The toxins of siphonophore nematocysts have been examined only for *Physalia* (Cystonectae), and it has not been possible to isolate single types of nematocysts (*e.g.*, Lane and Dodge, 1958; Burnett and Calton, 1977). Therefore, it is unknown if the nematocyst types of other siphonophores contain toxin. It is also unknown if nematocyst toxins are a liquid in the capsule which is expelled through the thread upon discharge (like a syringe) or if the toxins are associated with the surface of the thread such that they contact the prey all along its discharged length (see Tardent and Holstein, 1982). The above results suggest that very little toxin would be injected directly into the copepod. Perhaps toxin is released into the water immediately surrounding the prey or over the surface of the prey and is taken up through the respiratory surfaces, pores such as chemoreceptors, or thin exoskeleton at the joints in sufficient quantity to narcotize or kill the prey. The extent to which the experimental prey were entangled by nematocyst threads seemed adequate to immobilize them (Fig. 4F).

Cystonect siphonophores lack nematocysts with heavily-spined or club-shaped threads that adhere to the crustacean prey captured by calycophoran and physonect siphonophores. Therefore, cystonects may be unable to entangle crustaceans and may be limited to prey types that can be penetrated by their nematocysts. The isorhizas in cystonect tentacles may be unable to penetrate the exoskeleton of crustaceans; the isorhizas are firmly anchored in the tentacle (Cormier and Hessinger, 1980), and have no structure for puncturing comparable to the stylets of stenoteles. The results of this study cannot exclude the possibility that cystonect nematocysts are stimulated to discharge only by soft-bodied prey, perhaps by a mucus coating, which is lacking in crustaceans. Nematocysts of other coelenterates discharge upon a combination of chemical and mechanical stimulation (*e.g.*, Mariscal, 1974), and the stimulus can be quite specific in some cases (*e.g.*, Francis, 1973; Purcell, 1977). It is also possible that the toxins of cystonect nematocysts are not effective on crustaceans. However, toxin extracted from the nematocysts of *Physalia* (the only siphonophore thus tested) killed both crabs and fish when it was injected (Lane and Dodge, 1958; Burnett and Calton, 1977).

The above data show that there are marked differences in the tentacle nematocysts of calycophoran and physonect siphonophores, and those of cystonect siphonophores, as well as marked differences in diet. The evidence suggests that the different structure of the nematocyst threads could contribute to the dietary differences; the heavily-spined threads of calycophoran and physonect nematocysts may promote entanglement of crustacean prey, while the simple threads of cystonect nematocysts may promote penetration of soft-bodied prey.

Larger prey were captured by calycophoran and physonect siphonophore species with larger nematocysts. Nematocysts of larger volumes could contain longer threads, which presumably could be more effective in entangling prey. Nematocyst discharge may be due to an increase in the internal pressure of the capsule, caused by an influx of water in response to osmotic changes in the fluid of the capsule (Lubbock and Amos, 1981; Lubbock *et al.*, 1981). The force generated for discharge could be proportional to the volume of the capsule (greater for rounded nematocysts), or to the surface-to-volume ratio of the capsule (greater for elongate nematocysts) if the reactions were surface-mediated. This idea should be testable using methods of Tardent and Holstein (1982); presently, it is only speculation.

The nematocyst batteries of calycophoran and physonect siphonophores apparently function in two other capacities. The swimming activity of most siphonophores in these suborders serves to spread their tentacles in a 3-dimensional array (Mackie and Boag, 1963; Biggs, 1977) (Fig. 1). In *Muggiaea atlantica,* tentacle extension appears to be due to drag on the tentacles (Purcell and LaBarbera, unpub. results). Battery diameter is greater than tentacle diameter, hence the nematocyst batteries would increase drag at the ends of the tentacle branches and cause the tentacles to be drawn out. Thus, nematocyst batteries may aid in tentacle extension, enabling the spread of the tentacles to be greater and the tentacles finer (and therefore less conspicuous to prey) than possible with tentacles of uniform thickness.

Some information on morphology, behavior, and diet suggested that the nematocyst batteries of two siphonophore species may resemble small zooplankters and act to lure larger, predatory zooplankton into the siphonophore tentacles; the batteries of *Agalma okeni* resemble copepods, and some of *Athorybia rosacea* resemble fish larvae (Purcell, 1980). Further observations suggest that "lures" may occur in other siphonophore species. Two spots within the nematocyst batteries of *Nanomia cara* fluoresced when excited at 460–470 nm under an epifluorescence microscope (Purcell, unpubl. results), strongly suggesting that these spots are bioluminescent (see Morin and Reynolds, 1969). The batteries of *A. okeni, A. elegans, N. bijuga,* and *Forskalia* sp. did not fluoresce. Luminescent batteries may serve to attract prey at night or at depth, as may the luminescent lures (esca) of midwater anglerfish (Pietsch, 1974). The nematocyst batteries of *Athorybia lucida* reportedly resemble larvaceans in their houses actively pumping water (J. Trent, pers. comm.). The nematocyst batteries of *Physophora hydrostatica* began to vibrate upon the addition to the water of reduced glutathione (an inducer of feeding responses in hydrozoans, *e.g.,* Lenhoff and Schneiderman, 1959) (Purcell, unpub. results); motion of the lures of other organisms presumably serves to attract potential prey by visual or vibrational stimuli (Pietsch and Grobecker, 1978; Purcell, 1980). There are structures on the tentacles of *Rhizophysa filiformis* that lack nematocysts, but which have a red central spot that might attract the fish larvae prey of that species (Fig. 51). These observations suggest that luring of prey by nematocyst batteries may be an important and wide-spread phenomenon, and that morphological and behavioral mechanisms underlying prey selection are unexpectedly sophisticated.

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BIOMETRICAL AND HISTOLOGICAL ASPECTS OF THE REPRODUCTIVE CYCLE OF THE OVARIES OF *ASTERIAS RUBENS* (ECHINODERMATA)

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ABSTRACT

The reproductive cycle of the ovaries of the starfish *Asterias rubens* has been studied by histological and biometrical methods and can be divided into 4 stages, based on semi-quantitative and biometrical data. On the basis of biometrical data and their statistical analyses, each stage can be subdivided into two substages.

Frequency distributions of oocyte diameters present a characteristic for each (sub)stage. McCall units were calculated from the frequency distribution of oocyte diameters of stage 3. This stage is the reference for each other stage or substage and can be used as reference for any other group of animals of the species under examination. McCall transformations allow for the comparison of frequency distributions. It is concluded that the oocyte diameter is a reliable parameter for delineating the reproductive cycle and oogenesis of *Asterias rubens,* but a detailed description of the annual reproductive cycle required the analysis of a complex of variables, including the correlations among them.

From the stages' frequency distributions, the theoretical and proportional duration of each stage and of the period of spawning was derived. Minor, not significant variations have been found from year to year in the duration of the stages and in the beginning and conclusion of each period.

INTRODUCTION

Recently, steroidogenesis of ovaries and pyloric caeca of the starfish has been studied with some different steroids as precursor (Schoenmakers, 1979; Schoenmakers and Voogt, 1980, 1981). The steroid synthesizing capacity (Schoenmakers, 1980), and the presence and levels of endogenous steroids (Dieleman and Schoenmakers, 1979; Schoenmakers and Dieleman, 1981) have been determined during the reproductive cycle. Information on changes of the ovaries during the reproductive cycle will lead to a better understanding of these biochemical data.

Schoenmakers *et al.* (1981b) described the histology and ultrastructure of the ovaries of the starfish *Asterias rubens* and divided oogenesis into four phases: a) multiplication phase of oogonia, b) initial growth phase of oocytes I, c) growth phase proper of oocytes I, and d) post-growth phase of oocytes I. They also observed that the oocytes within an animal are seldom all in the same phase of oogenesis.

In several studies on the reproduction of echinoderms the question has been raised as to which criteria are the most appropriate for delineating the reproductive cycle. A number of studies on echinoderm reproductive cycles are based on the gonad index (Lasker and Giese, 1954) for measuring reproductive conditions (Feder, 1956; Farmanfarmaian *et al.*, 1958; Greenfield, 1959; Boolootian, 1966). Patent (1969), however,

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pointed out that the gonad index may give a useful measure of the relative size of the gonads, but fails to indicate the actual condition of the gametes. Moreover, the gonads of some echinoderms, such as *Gorgonocephalus caryi*, cannot be removed in total, so that a gonad index cannot be determined (Patent, 1969). Other authors applied periodic histological examinations of the gonads as a means of determining the reproductive cycle in echinoderms (Yoshida, 1952; Tanaka, 1958). Chia (1968) studied the cyclical changes of the oocytes of *Leptasterias hexactis* by measuring the diameter of both fresh and fixed material on a monthly basis. Pearse (1965) showed the oocyte changes of *Odontaster validus* with frequency polygons, averaged for all females of each sample. Patent (1969) measured the diameter of twenty of the largest oocytes from each female specimen and determined the mean. For *Asterias rubens* Vevers (1949) measured the length of the ovaries and the diameter of the oocytes; Kowalski (1955) determined the gonad index and the diameter of fresh oocytes, and Jangoux and Vloebergh (1973) composed frequency polygons of oocyte diameters and measured the gonad index.

Applying histological and biometrical methods, the present study deals with the analysis of a complex of variables, with which the reproductive cycle of *Asterias rubens* can be divided into stages and substages.

MATERIALS AND METHODS

Animals

Asterias rubens were collected from the Wadden Sea, east of the island of Texel (The Netherlands), at three week intervals during 2.5 years. The animals were kept in aerated sea water at 6°C for three days until processing. Ovaries of five females from each sample were studied with the light microscope. Only specimens with arms varying from 6.0 to 10.0 cm were used, the length measured from the center of the mouth along the oral face to the tip of the arm. Parasitized (parasite: *Orchitophyra stellarum*) animals were discarded.

Data on the course of quantitative parameters during the reproductive cycle, and semi-quantitative data were obtained from animals collected during 1 year ($n = 83$). The approximation of the duration of the stages of the reproductive cycle was based on data from all the collected animals ($n = 188$).

Dissection of animals

After cutting the arms laterally on both sides, the gonads were separated from the gonoduct. The animals were sexed by means of a squash preparation of gonadal tissue, which was also examined for the possible presence of parasites.

Histological procedure

Small pieces from the proximal, the middle, and the distal part of the ovaries were fixed in Bouin's fluid, embedded in paraffin, and sectioned at 7 μm. Sections were stained with Brookes' trichrome (Brookes, 1968) and Periodic Acid Schiff-Orange G (PAS/OG). Pilot studies showed the structure to be the same in the different parts of the ovaries. For this reason, detailed examinations were made only in cross-sections of the middle part of the ovaries.

In the Brookes-stained sections the percentages of pear-shaped oocytes and basophilic oocytes were determined and the presence or absence of the following characteristics was scored for each animal: dividing oogonia in the germinal epithelium, cell-debris, phagocytozing cells, follicle cells, jelly coat, cortical vesicles, degenerating oocytes, and "loose" oocytes. In the PAS/OG-stained sections the percentage of PAS-positive oocytes was determined and the presence or absence of invaginations of the hemal system and the amount of the hemal fluid was scored for each animal. The semi-quantitative scores have the following gradation: − = absent; ± = scarce; + = present; ++ = frequent; +++ = abundant.

Histometric procedure and processing of data

To obtain morphometric data on the variation of oocytes, the Brookes-stained sections of ovaries of each animal were used. Representative areas, containing at least 100 oocytes, were traced from the ovarian wall to the middle of the ovarian lumen, and drawn with a Zeiss microscope, equipped with camera lucida. The area of each of these oocytes was measured with a digitizer (Hewlett-Packard, type 9864A) in combination with a calculator (Hewlett-Packard, type 9820). Approximating the sectioned oocytes as circles, the diameters of these oocytes were calculated, averaged, and the 95% confidence interval (CI) was determined. Also the average (with CI) of the ten smallest oocyte diameters and of the ten largest oocyte diameters were computed for each animal. Finally, the median value of one hundred oocytes was determined for each animal.

These quantitative and also the semi-quantitative data were used for dividing the reproductive cycle into stages. First of all, the oocyte diameters of every single animal were compared with those of all other animals. For that purpose the one hundred oocyte diameters of one animal were pooled with those of another animal and arranged in order of ascending size to determine the common median. The presence or absence of differences between the two animals was then statistically analyzed by applying the median test for the 2 × 2 contingency table, according to Sokal and Rohlf (1969). This procedure was carried out for all animals. The specimens not showing significant differences in the oocyte diameters were placed in groups or stages. For each stage the frequency distribution of the oocyte diameter was determined. The symmetry of each distribution was checked, using the symmetry test (Scharf *et al.*, 1968). In case of asymmetry ($\chi^2 > 3.841$) McCall (1949) transformations were applied according to Scharf *et al.* (1968). The McCall units (T-values) were calculated from the frequency distribution of oocyte diameters of the stage that contains, with a range of 7–140 μm, almost all the possible diameters found in all stages or substages together. This frequency distribution was used as the reference, so that the calculated T-values, corresponding with diameters between 7 and 140 μm, could also be applied to corresponding diameters of each other stage or substage. The other T-values were found by interpolation or extrapolation via quadratic regression. The significance of differences between the transformed frequency distributions was determined by using the excentricity test. The oocyte diameters of each stage were also plotted as cumulative quantile, and next the semi-interquartile range was calculated. In addition, the other parameters were used to check this subdivision of the reproductive cycle. Therefore, the significance of differences between the data for the stages of this subdivision was determined for each parameter with the Student's *t*-test and the test of Wilcoxon for quantitative and semi-quantitative data, respectively.

Based on a comparison of the semi-quantitative data with the quantitative results and their statistical evaluations, it was possible to halve the number of stages by combining two subsequent stages at a time, starting from spawning. Thus the reproductive cycle can be divided into a number of stages, and each stage into two substages. For each stage, *i.e.*, combination of two substages, the frequency distribution and the cumulative quantile of the oocyte diameters were plotted and the semi-interquartile range was calculated. Following these procedures, the stage of the reproductive cycle is obtained for each animal separately. This made it possible to determine the maturation index, *i.e.*, the average stage per sample. Moreover, the proportional distribution of the stages per sample can be determined and this produces the frequency distribution of each stage. From the mean and standard deviation of the frequency distribution of the stages, the theoretical duration of the stages can be approximated, using 3× the standard deviation, right and left of the mean. The actual number of days of one reproductive cycle was defined as the number of days between the mean of the frequency distribution of a certain stage in one reproductive cycle and the mean of the frequency distribution of that stage in the following reproductive cycle. The ratio between this number of days and the sum of the theoretical durations of all stages of one reproductive cycle can be calculated. The proportional (relative) duration of each stage was computed from the theoretical duration by multiplying it with this ratio. Then, the excentricity of the proportional duration, its corresponding proportional area of the frequency distribution, and its tail-probability are to be calculated.

The course of quantitatively determined variables during the reproductive cycle also was studied. For that purpose the mean percentage (±SEM) per sample of pear-shaped oocytes and of the PAS-positivity and basophilia of the oocytes, the mean (±CI) per sample of the pyloric caeca index and of the gonad index, the weighed average per sample of the ten smallest, of the ten largest, and of one hundred oocyte diameters, and the mean (±SEM) per sample of the median values were calculated. These data (and the maturation indices) were plotted *versus* time. Correlation between the quantitatively determined variables was examined for the individual data using linear regression by the method of least squares and the correlation test.

Calculations and statistics

The diameters were calculated from the areas of the oocytes, approximating the sectioned oocytes as circles. The standard deviations (S.D.), the standard error of the mean (SEM) and the 95% confidence interval (CI) were calculated as usual.

The weighed averages of the average values for the animals per sample were determined with the formula:

$$\bar{x}_w = \frac{\sum w_i \times x_i}{\sum w_i} \pm \frac{1}{\sqrt{\sum w_i}}, \ (\bar{x}_w = \text{weighed average; } w_i = \frac{1}{(\mathrm{CI})^2}).$$

The maturation index was calculated according to Yoshida (1952) as:

$$\mathrm{MI} = \frac{\sum (n \times F)}{N}, \ (n = \text{number of animals in stages } F;$$

$$N = \text{total number of animals in the sample}).$$

The organ index was expressed as the percentage organ weight (fresh) of the total weight.

The semi-interquartile range (Q) was calculated with the formula:

$$Q = \frac{Q_3 - Q_1}{2}, \ (Q_1 = \text{value, below which 25\% of the values;}$$

$$Q_3 = \text{value, below which 75\% of the values}).$$

The median test for the 2 × 2 contingency table was performed using the equation:

$$\chi^2 = \frac{n(ad-bc)^2}{(a+b)(c+d)(a+c)(b+d)} \text{ (equation 16.9, Sokal and Rohlf, 1969).}$$

The differences between the oocyte diameters of two animals are not significantly different, if $\chi^2 < 3.841$ ($P = 0.05$).

The symmetry of the frequency distribution of oocyte diameters of each stage was tested according to Scharf *et al.* (1968) with the formula:

$$\chi^2 = \frac{(H^+ + H^-)^2}{n}$$

in which:

$H^+ = \sum y(x > \bar{x})$ = number of oocyte diameters larger than the mean

$H^- = \sum y(x < \bar{x})$ = number of oocyte diameters smaller than the mean

$n = \sum y$ = total number of oocytes diameters.

A frequency distribution is concluded to be asymmetric, if $\chi^2 > 3.841$ ($P = 0.05$).

McCall transformation

The method of transformation (McCall, 1949) is based on the determination of a correction factor for the abscissa. To that end ζ_m is calculated via the frequency of oocyte diameter (y_m) with the following formulas:

$$\eta_m = 0.5y_m + \sum_{N}^{m+1} y_i,$$

($i = 1, 2 \cdots N$; $m = 0, 1, 2 \cdots N$; N = number of frequencies).

$$\zeta_m = \frac{\eta_m + 100(\%)}{n}, \text{ (} n = \textstyle\sum y \text{ = total number of oocyte diameters).}$$

The McCall value T_m can be found in a table via ζ_m. Using a computer the McCall value T_m can be calculated via a function. Firstly, the factor u_m must be calculated via the formula:

$$u_m = \frac{\zeta_m - 1}{50}$$

T_m can be computed by using the function (the indices have been omitted):

$$T = a_1 - a_2 \cdot \operatorname{arctanh}(u) + a_3 \cdot \operatorname{arAmp}(u) + a_4 \cdot \tan(u)$$
$$+ a_5 \cdot \sinh(u) - a_6 \cdot \arcsin(u) + a_7 \cdot (u)^3,$$

in which:

$a_1 = 49.99893727$ $a_5 = 639.378889$

$a_2 = 4.32564762$ $a_6 = 28.6845627$

$a_3 = 827.489609$ $a_7 = 34.2207938$

$a_4 = 208.528568$

RESULTS

By using the median test, statistical analysis of the oocyte diameters produced eight significantly different groups of animals. The animals belonging to a certain group are not significantly different from each other ($\chi^2 < 3.841$). On the basis of these results the annual reproductive cycle of *Asterias rubens* can be subdivided into eight substages. Essential data of the frequency distributions of the oocyte diameters for these substages before and after McCall (1949) transformation are summarized in Table I. Differences between them appear to be significant in all cases ($P < 0.0001$). This confirms the subdivision of the reproductive cycle into eight substages. Based on this subdivision, the significance of differences between the data for the substages was determined for each parameter, using the Student's *t*-test or the test of Wilcoxon. The median values and the averages of the one hundred oocytes diameters also permit us to distinguish between eight substages ($P < 0.005$; Fig. 1). However, a comparison of these results with the other parameters, especially the combination of the semi-quantitative data, provides arguments in support of reducing the eight substages to four stages, by combining two subsequent substages at a time, starting from spawning. Consequently, the annual reproductive cycle can be divided into four stages, each of which can be subdivided into two substages, on the basis of the biometrical results.

As to the other quantitative data, the averages of the ten largest oocytes determine the substages 1a and 1b, 2a and 2b, and 3a and 3b ($P < 0.005$), the averages of the ten smallest oocytes the substages 3a and 3b, and 4a and 4b ($P < 0.005$); the percentage

TABLE I

The oocyte diameters of specimens of Asterias rubens *in the eight substages and four stages of the annual reproductive cycle*

Substage	Number of specimens	Oocyte diameter (μm)	Symmetry[1]	Transformed oocyte diameter (McCall units)	Symmetry[2]
1a	14	23.31 ± 0.19	−	29.28 ± 0.15	+
1b	12	27.16 ± 0.21	−	32.07 ± 0.15	−
2a	7	36.33 ± 0.41	+	37.13 ± 0.19	− ($\chi_x^2 < \chi_T^2$)
2b	7	42.23 ± 0.49	+	39.62 ± 0.23	+
3a	13	60.20 ± 0.62	+	47.07 ± 0.25	+
3b	12	74.52 ± 0.65	+	52.81 ± 0.26	+
4a	10	92.95 ± 0.70	−	60.25 ± 0.28	− ($\chi_x^2 > \chi_T^2$)
4b	8	102.02 ± 0.56	−	63.93 ± 0.24	+
Stage					
1	26	25.23 ± 0.15	−	30.67 ± 0.11	+
2	14	39.72 ± 0.37	−	38.53 ± 0.18	− ($\chi_x^2 > \chi_T^2$)
3	25	67.51 ± 0.49	+	50.00 ± 0.20[3]	+
4	18	97.55 ± 0.49	−	62.13 ± 0.20[4]	− ($\chi_x^2 > \chi_T^2$)

[1] Symmetry of the frequency distribution of oocyte diameters before McCall transformation.

[2] Symmetry of the frequency distribution of oocyte diameters after McCall transformation.

[3] The McCall units (T-values) were calculated from the frequency distribution of oocyte diameters of stage 3. Since the frequency distribution, with a range of 7–140 μm, contains nearly all the possible oocyte diameters found in all stages or substages together, it is used as reference for each other stage or substage. Therefore, the calculated T-values, corresponding with diameters between 7 and 140 μm, are also applied to corresponding diameters of each other stage or substage.

[4] Results expressed as means ± SEM.

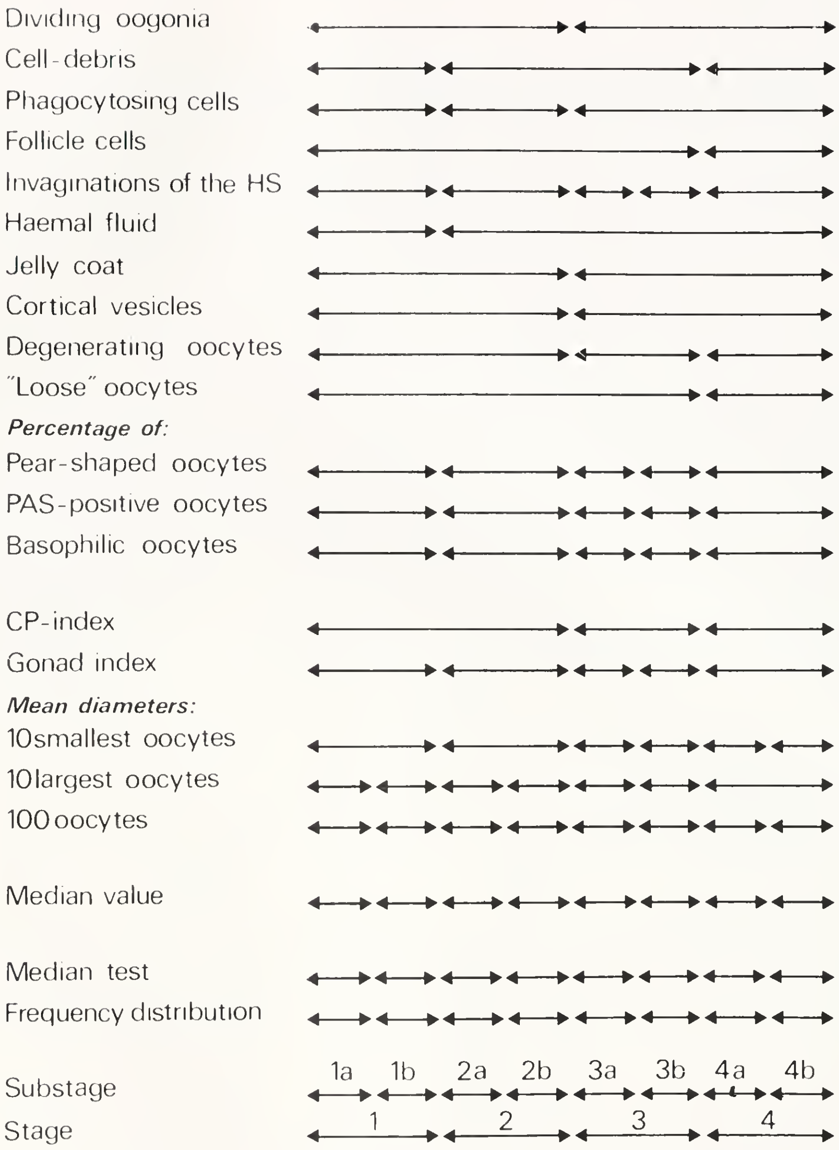
Dividing oogonia
Cell-debris
Phagocytosing cells
Follicle cells
Invaginations of the HS
Haemal fluid
Jelly coat
Cortical vesicles
Degenerating oocytes
"Loose" oocytes
Percentage of:
Pear-shaped oocytes
PAS-positive oocytes
Basophilic oocytes
CP-index
Gonad index
Mean diameters:
10 smallest oocytes
10 largest oocytes
100 oocytes
Median value
Median test
Frequency distribution
Substage
1a
1b
2a
2b
3a
3b
4a
4b
Stage
1
2
3
4

FIGURE 1. Summary of parameters and for each an outline of significant changes, indicated by arrows, from (sub)stage to (sub)stage during the annual reproductive cycle of *Asterias rubens.* HS: hemal system; CP: pyloric caeca.

of pear-shaped oocytes, the percentages of PAS-positivity and basophilia of oocytes, and the gonad index together define the substages 3a and 3b ($P < 0.005$).

Dividing oogonia in the germinal epithelium, cell-debris, phagocytozing cells, and follicle cells are shown in Plate I. Invaginations of the hemal system are depicted in Plate II, 1 and 2, the hemal fluid in Plate II, 3 and 4. The jelly coat and cortical vesicles of full-grown oocytes are visible in Plate III, 1 and 2, respectively. Examples

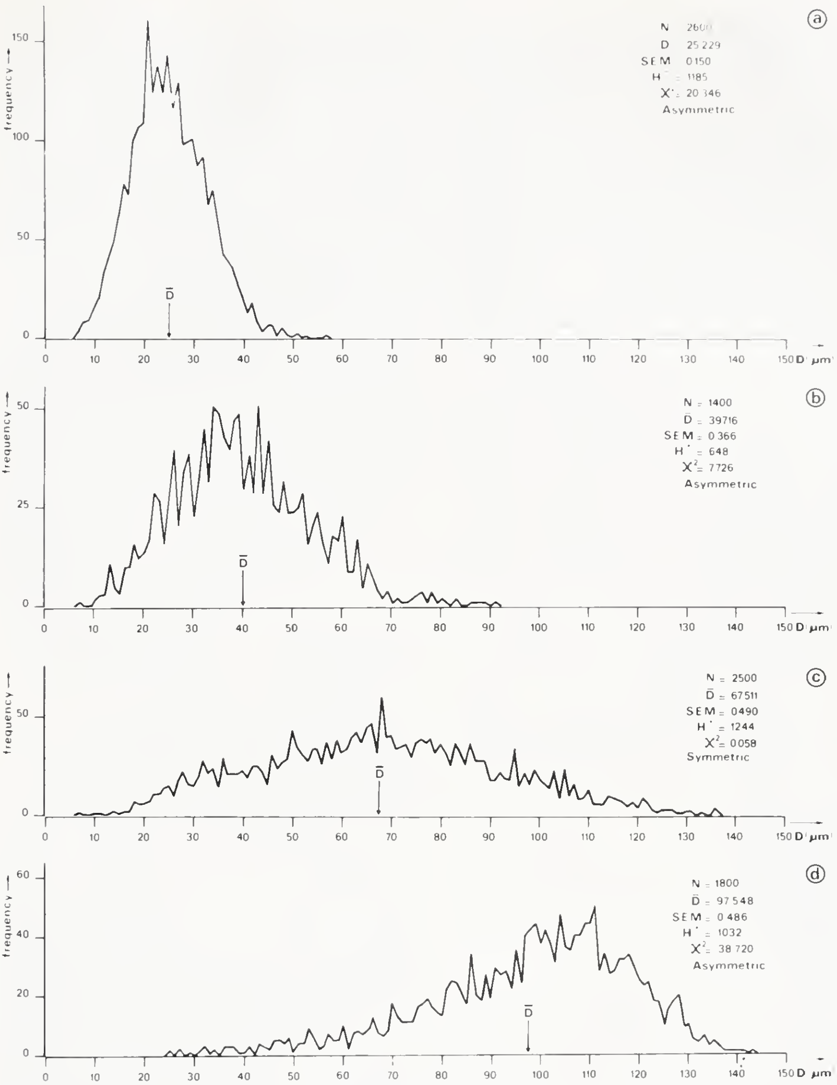

FIGURE 2. Frequency distributions of oocyte diameters of *Asterias rubens* (before McCall transformation). a: For the resting stage (stage 1; substages 1a and 1b); b: for the early growth stage (stage 2; substages 2a and 2b); c: for the stage of late growth and partial maturation (stage 3; substages 3a and 3b); d: for the maturation stage (stage 4; substages 4a and 4b).

of degenerating and "loose" oocytes are presented in Plate III, 3 and 4. All these parameters have been semi-quantitatively scored for each animal. The semi-quantitative data, obtained in this manner, do not support a subdivision into eight substages. The individual semi-quantitatively determined parameters hardly justify four stages. Only the decrease in the number of invaginations of the hemal system shows four stages ($P < 0.025$) and permits stage 3 to be subdivided into two substages ($P < 0.05$). The absence of dividing oogonia and the presence of the jelly coat, cortical vesicles and degenerating oocytes define stages 3 and 4 ($P < 0.025$, $P < 0.005$, $P < 0.005$, P

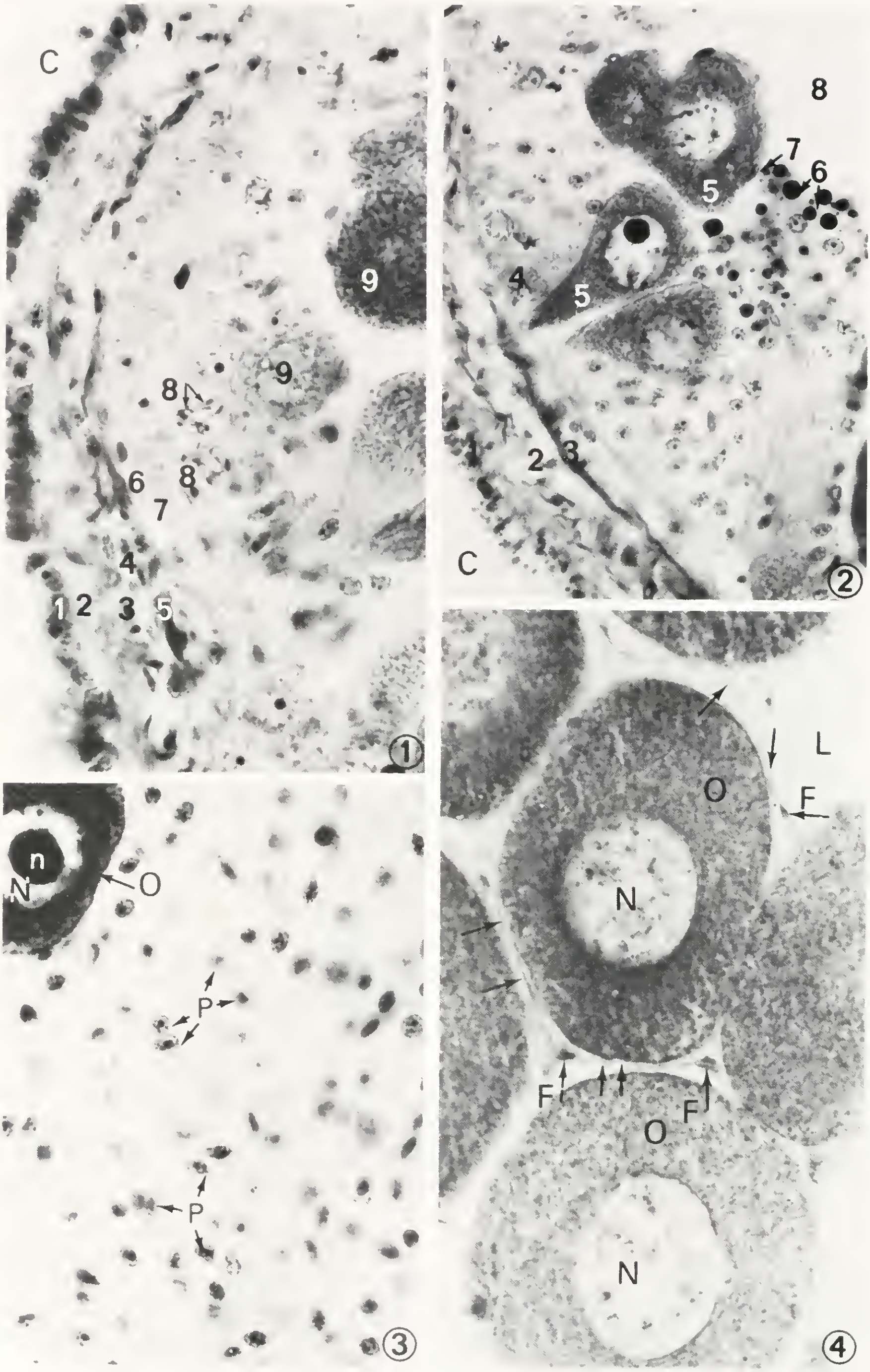
C
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PLATE I: 1. Detail of an ovarian wall of *Asterias rubens*. Brookes' trichrome staining. ×820. C: Coelom; 1: perivisceral coelomic epithelium; 2: connective tissue; 3: layer with outer genital coelomic epithelium and muscle cells; 4: genital coelomic sinus; 5: layer with inner genital coelomic epithelium and muscle cells; 6: hemal system; 7: germinal epithelium; 8: dividing oogonia; 9: oocytes.

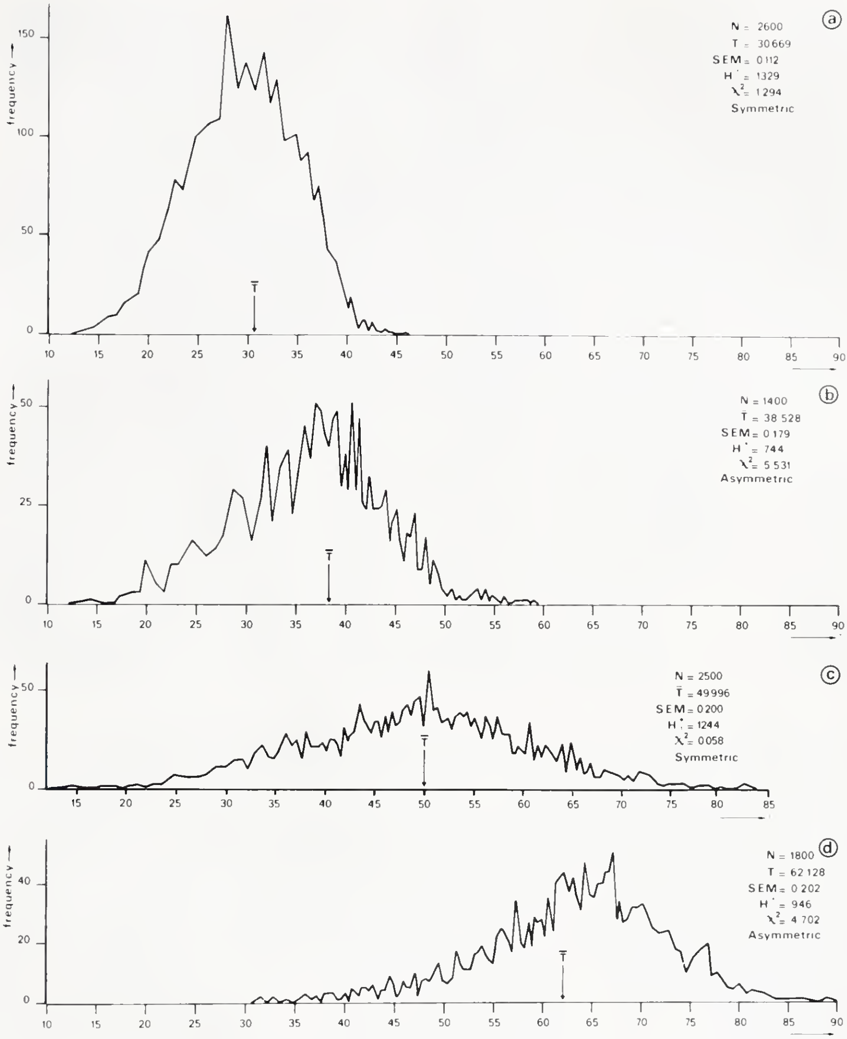

FIGURE 3. Frequency distributions of oocyte diameters of *Asterias rubens* (after McCall transformation). a: for the resting stage (stage 1; substages 1a and 1b); b: for the early growth stage (stage 2; substages 2a and 2b); c: for the stage of late growth and partial maturation (stage 3; substages 3a and 3b); d: for the maturation stage (stage 4; substages 4a and 4b).

< 0.005, respectively) and the increase of degenerating oocytes distinguishes stage 3 from stage 4 ($P < 0.05$). The amount of cell-debris separates stage 1 and stage 4 from the others ($P < 0.005$, $P < 0.005$, respectively); the number of phagocytozing cells

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2. Detail of an ovary. Brookes' trichrome staining. ×520. C: Coelom; 1: outer part of the ovarian wall; 2: genital coelomic sinus; 3: inner part of the ovarian wall; 4: germinal epithelium; 5: oocytes; 6: cell-debris; 7: follicle cells; 8: ovarian lumen.
3. Detail of an ovarian lumen. Brookes' trichrome staining. ×820. P: Phagocytozing cells; O: oocyte; N: nucleus; n: nucleolus.
4. Detail of an ovarian lumen. Brookes' trichrome staining. ×520. L: Ovarian lumen; O: oocytes, surrounded by follicle cells (F); N: nucleus. Note the long cellular processes (arrows) of the follicle cells around the oocytes.

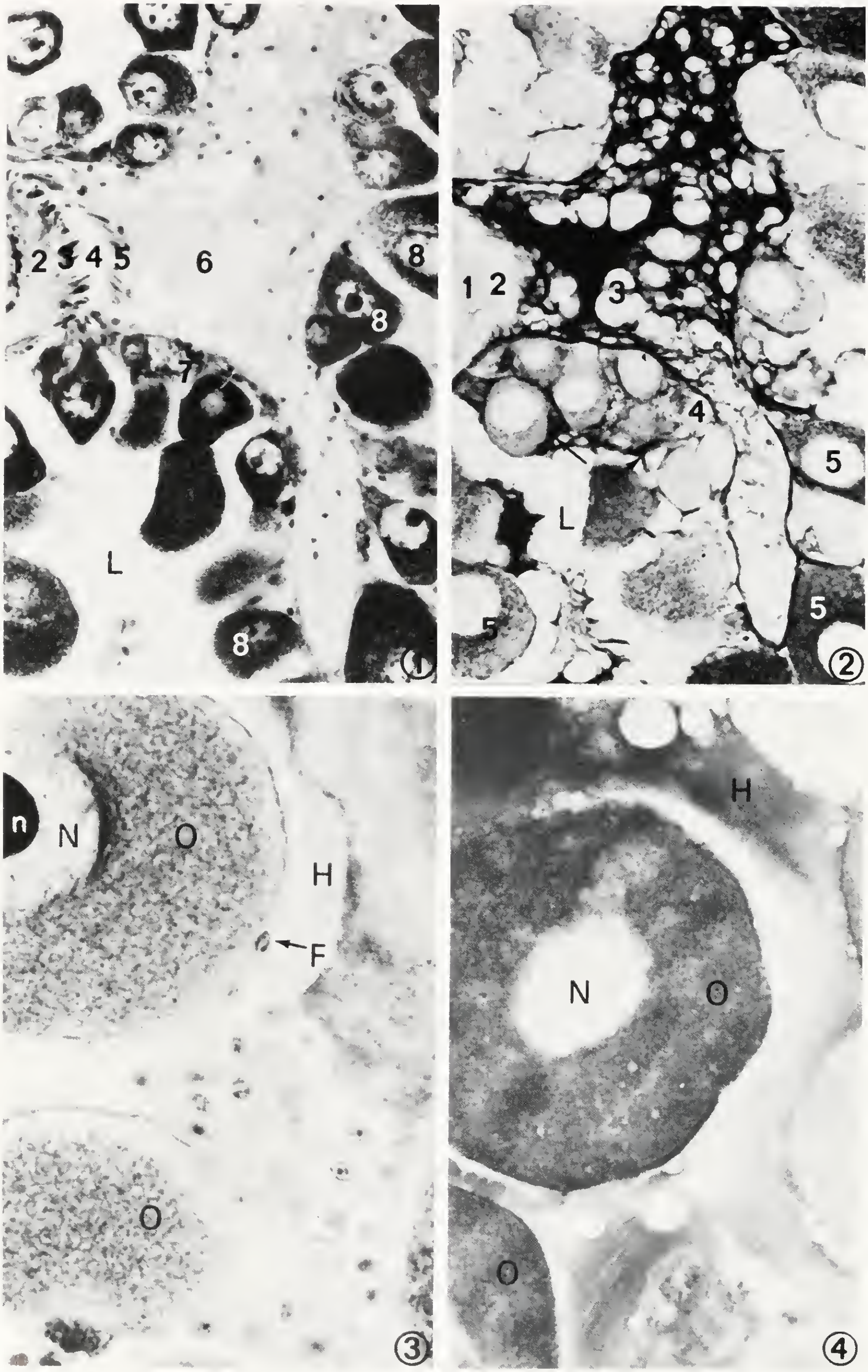

PLATE II: 1. Detail of an ovary of *Asterias rubens.* Brookes' trichrome staining. ×325. 1: Epithelium; 2: connective tissue; 3: layer with epithelium and muscle cells; 4: genital coelomic sinus; 5: layer with epithelium and muscle cells; 6: hemal system with invaginations into the ovarian lumen (L); 7: germinal epithelium; 8: oocytes. Note the close contact between the hemal system and the oocytes.

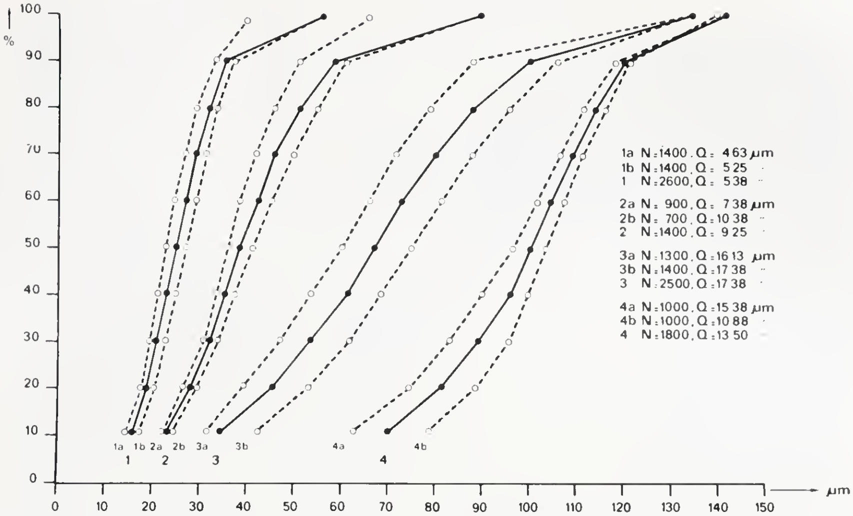

FIGURE 4. Cumulative quantiles of oocyte diameters for the stages and substages of the annual reproductive cycle of *Asterias rubens*. O – – – O: Cumulative quantiles of oocyte diameters from animals belonging to a substage, mutually not significantly different ($\chi^2 < 3.841$). ● —— ●: Cumulative quantiles of oocyte diameters from animals belonging to two subsequent substages, and in the same stage of the annual reproductive cycle. Q: Semi-interquartile range.

distinguishes stage 1 from stage 2 ($P < 0.005$), and stage 2 from stages 3 and 4 ($P < 0.025$). The absence of the hemal fluid in stage 1 distinguishes this stage from the others ($P < 0.005$). Finally, the desintegration of follicle cells and the presence of "loose" oocytes indicates a difference between stage 4 and the other stages ($P < 0.005$, $P < 0.005$, respectively).

For the four stages, obtained in this manner, the frequency distributions of oocyte diameters before and after McCall (1949) transformation are given in Figures 2 and 3, respectively. Essential data of these frequency distributions are summarized in Table I. Differences between these distributions are significant ($P < 0.0001$). For each stage and each substage the cumulative quantile of the oocyte diameters has been plotted (Fig. 4).

Determining the proportional distribution of the stages per sample produces the frequency distributions of the stages of the reproductive cycle, presented in Figure 5. Some of the frequency distributions are slightly but significantly asymmetric. In approximating the duration of the stages, this asymmetry is neglected. Differences between the frequency distributions of the stages within one reproductive cycle were found to be significant ($P < 0.0001$). The frequency distributions of the stages of one reproductive cycle are different from those of the following cycle, but the differences proved to be not significant. The frequency distribution of each stage and the dis-

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2. Parallel section to Part I. PAS/OG staining. ×325. 1: Outer part of the ovarian wall; 2: genital coelomic sinus; 3: the hemal system, PAS-positive, with invaginations into the ovarian lumen (L), branching retiformly between the oocytes (arrows); 4: germinal epithelium; 5: oocytes.

3. Detail of an ovarian lumen. Brookes' trichrome staining. ×820. O: Oocytes; N: nucleus; n: nucleolus; F: follicle cell; H: hemal fluid between the oocytes.

4. Detail of an ovarian lumen. PAS/OG staining. ×520. O: Oocytes; N: nucleus; H: hemal fluid, a mucoid-like and PAS-positive substance between the oocytes.

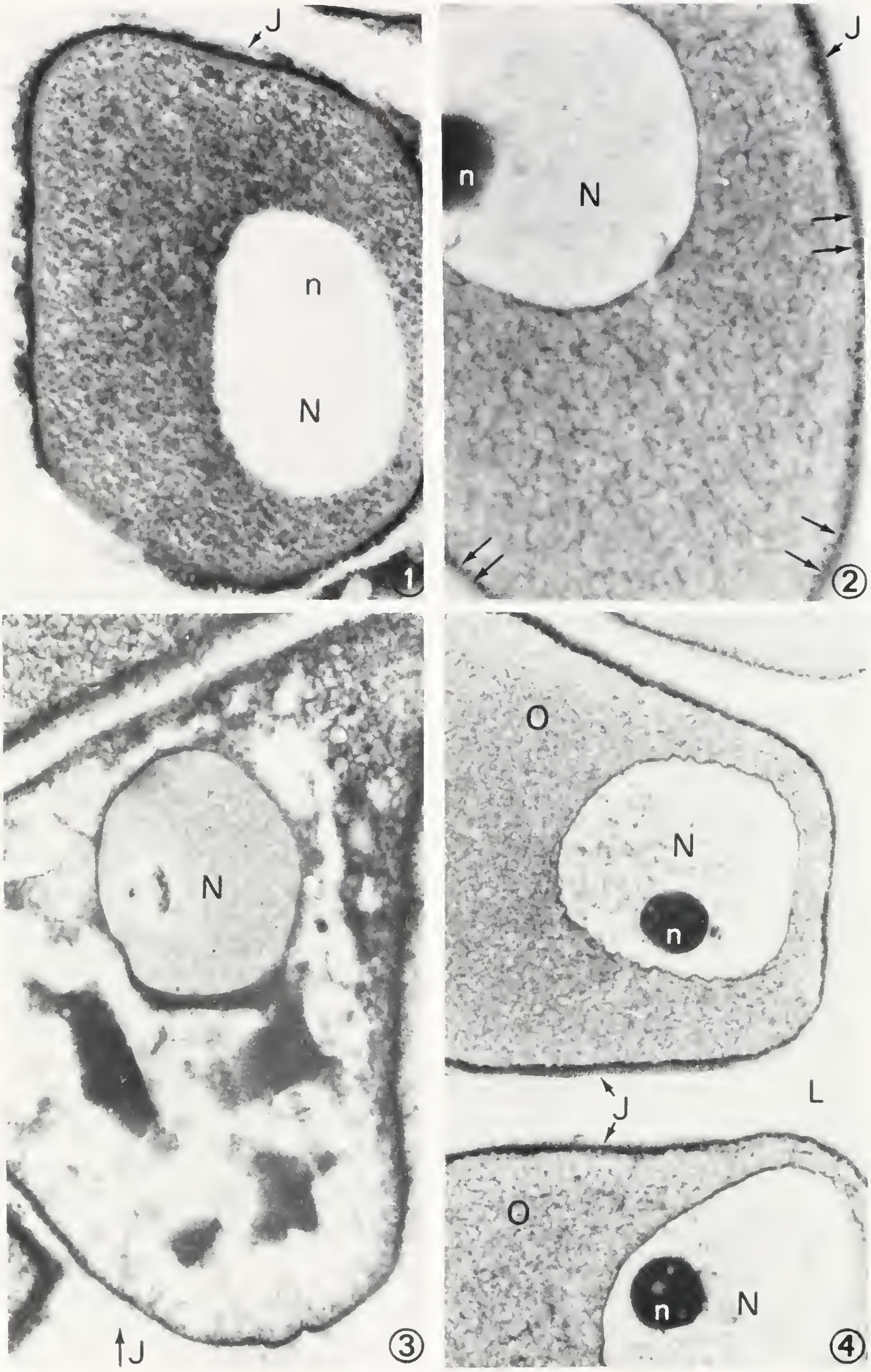

PLATE III: 1. Section of a full-grown oocyte of *Asterias rubens*. PAS/OG staining. ×520. N: Nucleus; n: nucleolus; J: jelly coat.

2. Detail of a full-grown oocyte. Brookes' trichrome staining. ×820. N: Nucleus; n: nucleolus; J: jelly coat. Note the cortical vesicles (arrows).

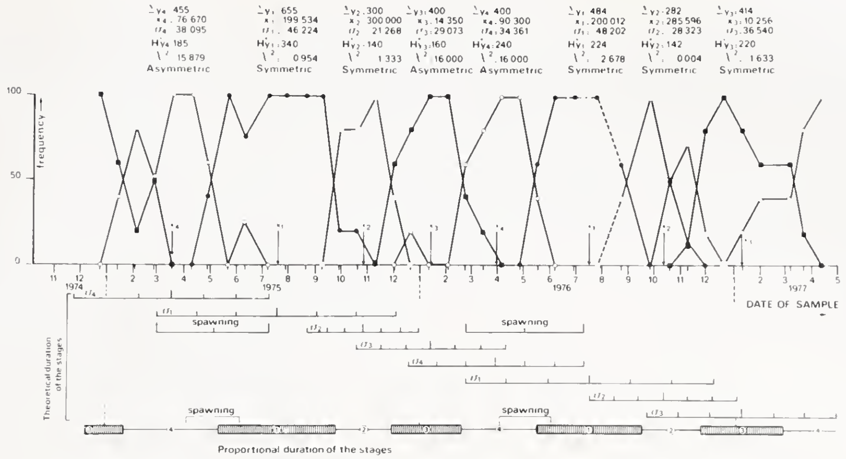

FIGURE 5. Frequency distribution of stages of the annual reproductive cycle of *Asterias rubens* during the period December 1974 to April 1977 (● —— ●: stage 1; □ —— □: stage 2; ■ —— ■: stage 3; ○ —— ○: stage 4); approximation of the theoretical and proportional duration of the stages (see: Tables II and III).

tributions of its substages were not significantly different, and the same applies to the distributions of the substages.

Essential data (the mean and the standard deviation) of the frequency distributions of the stages are summarized in Table II (columns 2 and 3). These data permit an approximation of the theoretical and the proportional duration of each stage, the excentricity of the proportional duration, its corresponding proportional area of each frequency distribution and each tail-probability. These results are given in Table II (columns 4 to 9).

Spawning takes place when the ovaries pass from stage 4 into stage 1. Thus, the spawning period can be defined as the period in which part of the population is in stage 4 and another part in stage 1. The theoretical duration of the spawning period is identical with the theoretical overlap of stage 4 and stage 1. Assuming a normal distribution of spawning animals, the mean and the standard deviation of this distribution can be found, just as the proportional duration of the spawning period, the proportional area and the tail-probability. Data on the spawning period are included also in Table II.

Figure 5 shows the theoretical duration of each stage and the spawning period. The theoretical and proportional duration of each stage, expressed by dates, are given in Table III. As can be noticed from this table, small gaps and overlaps are present between the proportional periods of the stages. Correction gives a continuous sequence of the periods of the stages in time. These "corrected" proportional durations of the stages are presented also in Table III and indicated in Figures 5, 6, and 7.

The proportional duration of stage 1 is the largest one (4 to 4.5 months), followed by stage 4 (3 to 3.5 months), stage 3 (2.5 to 3 months), and stage 2 (2 to 2.5 months).

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3. Section of a degenerating oocyte. Brookes' trichrome staining. ×520. N: Nucleus; J: jelly coat. Note the degenerating cytoplasm of the oocyte.

4. Section of "loose" oocytes. Brookes' trichrome staining. ×520. N: Nucleus; n: nucleolus; J: jelly coat; the oocytes (O) in the ovarian lumen (L) are free from each other. Note the absence of follicle cells and of hemal fluid.

TABLE II

The mean ($\bar{x}$) and the standard deviation (σ) of the frequency distribution of the stages of the annual reproductive cycle of Asterias rubens, *and from these data approximated durations of the stages and the proportional area of the frequency distributions*

Stage	$\bar{x}$	σ	Theoretical duration	Proportional duration	$x - \bar{x}$	$\frac{x - \bar{x}}{\sigma} = u$	Proportional area (one-tailed)	*P* (one-tailed)
Year 1								
4	76.67	38.10	(2×) 114.3	(2×) 54	54	1.4175	0.4218	0.0782
1	199.53	46.22	277.3	132	66	1.4278	0.4233	0.0767
2	300.00	21.27	127.6	61	30.5	1.4341	0.4242	0.0758
3	14.35	29.07	174.4	83	41.5	1.4274	0.4233	0.0767
4	90.30	34.36	(2×) 103.1	(2×) 49	49	1.4260	0.4231	0.0768
Total of days	378.63		796.7	379				
Spawning	126.50	21.83	131	62	31	1.4198	0.4222	0.0778
Year 2								
4	90.30	34.36	(2×) 103.1	(2×) 44	44	1.2805	0.3998	0.1002
1	200.01	48.20	289.2	123	61.5	1.2759	0.3990	0.1010
2	285.60	28.33	170.0	72	36	1.2708	0.3981	0.1019
3	10.26	36.54	(2×) 109.6	47	47	1.2863	0.4008	0.0992
Total of days	285.96		671.9	286				
Spawning	124.00	23.00	138	59	29.5	1.2826	0.4002	0.0998

The quantitative data were averaged per sample and plotted *versus* time, giving the course of a number of parameters during the annual reproductive cycle. The mean percentage (±SEM) per sample of pear-shaped oocytes and of the PAS-positivity and basophility of the oocytes, the average (±CI) per sample of the pyloric caeca index and of the gonad index, the weighed average per sample of the ten smallest,

TABLE III

The duration of the stages of the annual reproductive cycle of Asterias rubens, *expressed as dates*

Stage	Middle of the period	Theoretical duration		Proportional duration		"Corrected" proportional duration	
		from	to	from	to	from	to
4	03–18	11–23	07–10	01–23	05–11	01–22	05–12
1	07–19	03–02	12–04	05–14	09–23	05–12	09–25
2	10–27	08–24	12–30	09–26	11–26	09–25	11–28
3	01–14	10–19	04–11	12–03	02–24	11–28	02–19
4	03–30	12–18	07–11	02–15	05–13	02–19	05–17
1	07–18	02–24	12–10	05–17	09–17	05–17	09–15
2	10–12	07–19	01–05	09–06	11–16	09–15	11–24
3	01–10	09–23	04–30	11–24	02–26	11–24	02–26
Spawning 1st year	05–06	03–02	07–10	04–05	06–06		
Spawning 2nd year	05–03	02–24	07–11	04–04	06–02		

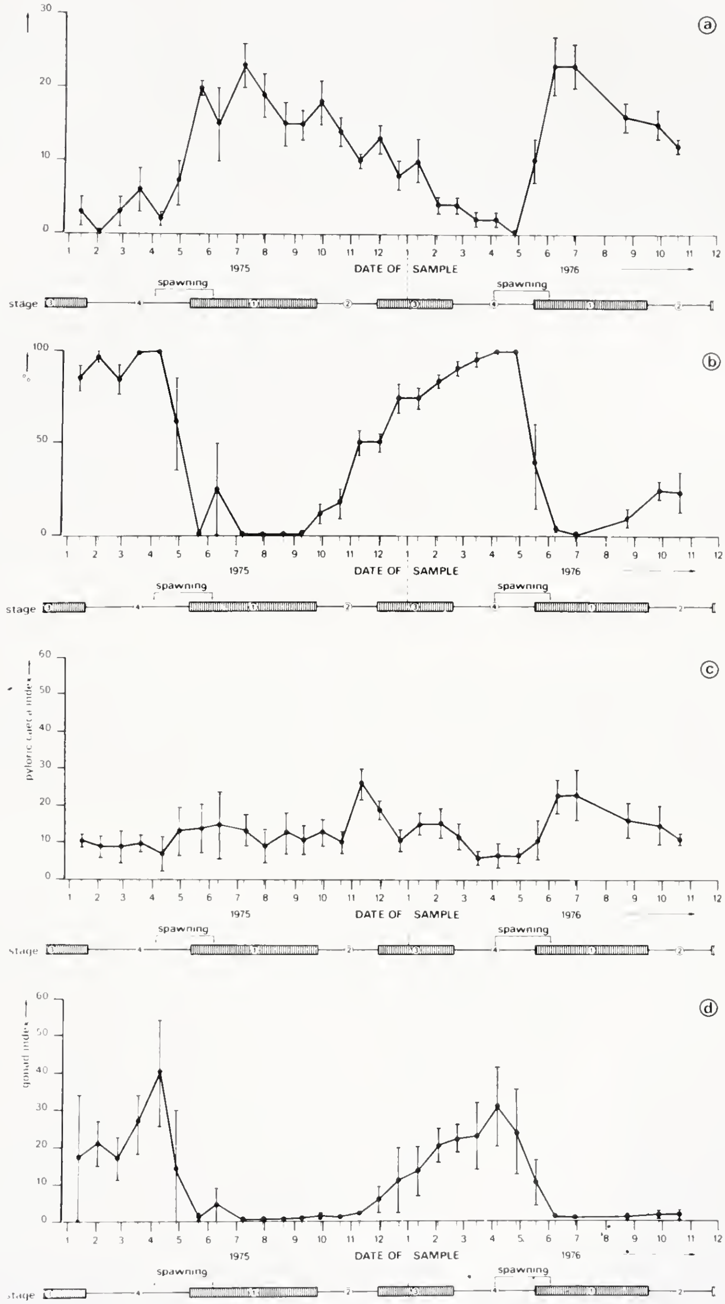

FIGURE 6. a: Mean percentage (±SEM) per sample of pear-shaped oocytes, b: mean percentage (±SEM) per sample of PAS-positivity and basophility of the oocytes, c: average per sample of the pyloric caeca index (±CI) and d: average per sample of the gonad index (±CI) during the annual reproductive cycle of *Asterias rubens*.

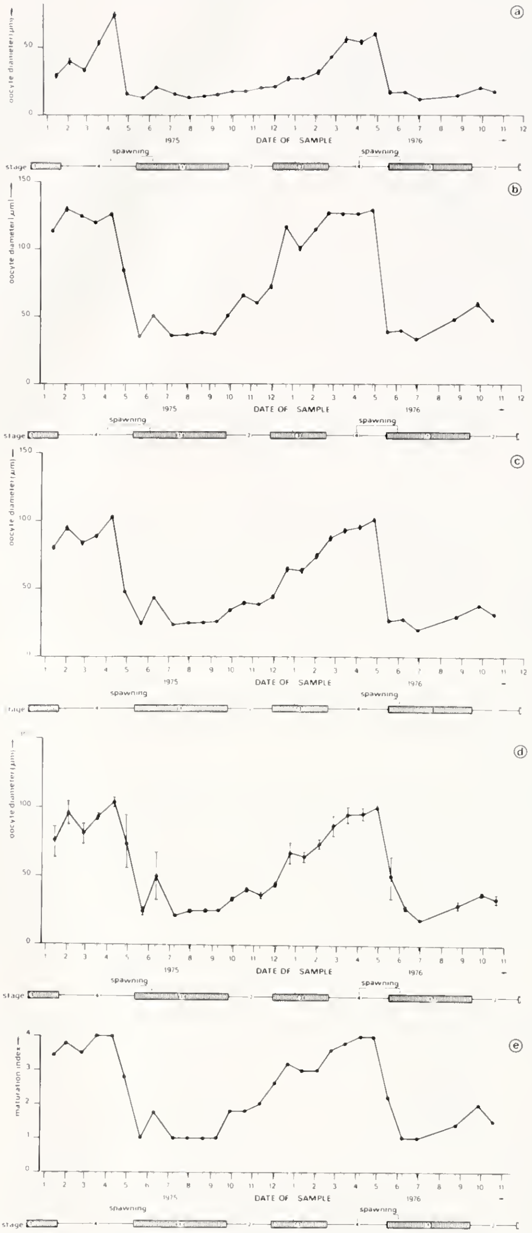

FIGURE 7. a: Weighed average per sample of the ten smallest oocyte diameters (in µm), b: weighed average per sample of the ten largest oocyte diameters (in µm), c: weighed average per sample of hundred oocyte diameters (in µm), d: average per sample of the median oocyte diameter (in µm), and e: maturation index per sample during the annual reproductive cycle of *Asterias rubens*.

of the ten largest, and of the one hundred oocyte diameters, and the mean (±SEM) per sample of the median values during the annual reproductive cycle of *Asterias rubens* are presented in Figures 6 and 7. The course of the maturation index per sample during the annual reproductive cycle is shown in Figure 7e.

The correlations between the quantitatively determined variables (number of animals = 83), expressed as correlation coefficients (r), are presented in a correlation matrix (Table IV). The median, the mean diameter of the one hundred oocytes, of the 10 largest oocytes, and of the 10 smallest oocytes, the percentage of PAS-positive and of basophilic oocytes, and the gonad index all show a significant, strongly positive correlation ($+0.798 < r < +0.998$; $P < 0.005$). These parameters are negatively correlated with the percentage of pear-shaped oocytes, and the correlation is significant ($-0.860 < r < -0.706$; $P < 0.005$). The pyloric caeca index is significantly and negatively correlated with the median, the mean diameter of the one hundred oocytes and of the 10 smallest oocytes, the gonad index ($-0.369 < r < -0.309$; $P < 0.005$), and with the mean diameter of the ten largest oocytes ($r = 0.240$; $0.01 < P < 0.025$); no significant correlation was found between the pyloric caeca index and the percentage of PAS-positive and basophilic oocytes, and the percentage of the pear-shaped oocytes.

Two groups of semi-quantitatively scored parameters can be distinguished. The parameters of group 1 are: dividing oogonia, cell-debris, phagocytozing cells, follicle cells, and invaginations of the hemal system; parameters of group 2 are: hemal fluid, jelly coat, cortical vesicles, degenerating oocytes, and "loose" oocytes. The parameters of group 1 are more or less positively correlated with each other and negatively correlated with the parameters of group 2, which are positively correlated with each other again.

DISCUSSION

The results prove that the reproductive cycle of *Asterias rubens* is an annual cycle, which can be divided into four stages, each comprised of two substages, making a total of eight substages. The subdivision into eight substages is based on the median test, applied to the oocyte diameters of all individual animals, and on the significant differences between the frequency distributions of the oocyte diameters per substage.

TABLE IV

Correlation matrix for the quantitative data obtained from 83 specimens of Asterias rubens *in different stages of the annual reproductive cycle.*

	1	2	3	4	5	6	7	8
1	1.000	0.998	0.959	0.945	0.877	0.910	−0.817	−0.310
2	0.998	1.000	0.969	0.949	0.878	0.913	−0.822	−0.309
3	0.959	0.969	1.000	0.960	0.826	0.798	−0.819	−0.240*
4	0.945	0.949	0.960	1.000	0.820	0.806	−0.860	−0.159**
5	0.877	0.878	0.826	0.820	1.000	0.833	−0.718	−0.312
6	0.910	0.913	0.798	0.806	0.833	1.000	−0.706	−0.369
7	−0.817	−0.822	−0.819	−0.860	−0.718	−0.706	1.000	0.128**
8	−0.310	−0.309	−0.240*	−0.159**	−0.312	−0.369	0.128**	1.000

[1] The median values.
[2] The mean diameter of the one hundred oocytes.
[3] The mean diameter of the ten largest oocytes.
[4] The percentage of PAS-positive and basophilic oocytes.
[5] The gonad index.
[6] The mean diameter of the ten smallest oocytes.
[7] The percentage of pear-shaped oocytes.
[8] $P < 0.005$. The pyloric caeca index.
* $0.01 < P < 0.025$.
** Not significant.

Also the median values and the averages of the one hundred oocytes support a subdivision into eight substages. This subdivision, based on biometrical data, may be indispensible in studying special aspects, such as vitellogenesis or the influence of endogenous and exogenous factors on the growth of oocytes.

The division of the annual reproductive cycle into four stages is primarily based on the combined semi-quantitative data, but also on a number of quantitatively determined criteria. This division may be useful in studying physiological problems such as energy metabolism and nutrition.

Frequency distributions of oocyte diameters present a characteristic for each stage or substage. Scharf *et al.* pointed out, that normal distribution of variables in morphometry is an exception and recommended to normalize non-normality, if the samples are large ($n > 80$) by McCall transformation. Fichna and Malendowicz (1975) also performed the McCall (1949) transformation according to Scharf *et al.* (1968) in their karyometric studies of the effects of gonadotropin and testosterone on gonadal tissues of the rat.

The McCall (1949) transformation allowed for comparison between the frequency distributions. Since the T-values were calculated from the frequency distribution of oocyte diameters of stage 3, this stage is the reference for each other stage or substage. By applying these calculated T-values to corresponding oocyte diameters of any other experimental group of animals, it is possible to compare the frequency distribution of oocyte diameters of this group with those of the stages and substages of the annual reproductive cycle presented in this study. Schoenmakers *et al.* (1981a) reported such a comparison with control and treated animals in their study on the effects of oestradiol-17β on the ovaries of the starfish *Asterias rubens.*

The frequency distributions of the stages also support a division into four stages since the frequency distributions of the substages of any one stage do not significantly differ from each other, nor from that of that stage, meaning that the substages are completely synchronic. The theoretical duration of the stages shows overlaps. Thus, parts of the four stages are synchronic. By approximating the proportional durations of the stages, a chronological sequence of the stages during the annual reproductive cycle was found. These results make it possible to predict, with a one side tail-probability of 8–10%, the period of the year in which 80–85% of the starfish population is in any one stage of the annual reproductive cycle. The duration of the stages and the time when a stage begins and ends probably will vary slightly from year to year. These variations are probably affected by the availability and amount of food, and by climatic conditions, especially temperature. Since the variations were found to be not significant, it may be concluded that, within the mentioned limits, the proportional durations of the stages and their chronological sequence in the annual reproductive cycle appear to be reproducible from year to year.

In the present study a number of variables have been studied quantitatively. By measuring the diameters of one hundred oocytes in representative areas of the ovaries, information was obtained, which could be processed for different purposes. Statistical analysis of the data indicated the presence or absence of differences between individual animals and made it possible to distinguish eight substages. The mentioned data formed the basic material for the frequency distributions of oocyte diameters, thus presenting a characteristic for each substage. Also the median values, the averages of the one hundred oocytes, of the ten smallest, and of the ten largest oocytes were calculated. The first two variables, both giving a characteristic of the oocytes in general, support the subdivision into eight substages. The last two variables show a more differentiated picture of the actual condition, in other words the oocytes in each animal are seldom simultaneously in the same phase of oogenesis.

The averages of the ten smallest oocytes, which may be less than 20 μm even in stage 3, demonstrate that only at the end of the reproductive cycle are all oocytes in the same phase of oogenesis. Therefore, it can be concluded that the oocyte diameter is a reliable parameter for delineating the reproductive cycle and oogenesis.

Other variables, based on the oocytes proper, are typical for the actual condition of the growing oocytes. For instance, PAS-positivity and basophilia, which are absent in stage 1, determine the course of vitellogenesis; the jelly coat, cortical vesicles, and "loose" oocytes indicate the maturation of oocytes. Moreover, other cells or features, related to the development of oocytes, *e.g.*, the presence of follicle cells, invaginations of the hemal system, and the hemal fluid, are characteristic for the stages of the annual reproductive cycle. The gonad index and most of the other quantitative data show a significant and negative correlation with the pyloric caeca index. It may be concluded that substances needed for vitellogenesis and growth of the oocytes are transported from the pyloric caeca to the ovaries.

This detailed description of the annual reproductive cycle of *Asterias rubens*, including a clearly defined subdivision in stages and substages, required the analysis of a complex of variables. However, a broad outline of the annual reproductive cycle can be carried out with a few variables only, since correlations were found between the quantitatively determined variables and between the semi-quantitative data. Indeed, one variable (for example, the gonad index) may be sufficient.

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ABNORMAL EGG STALK MORPHOLOGY IS CORRELATED WITH CLUTCH ATTRITION IN LABORATORY-MAINTAINED LOBSTERS (*HOMARUS*)

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ABSTRACT

Female lobsters attach fertilized eggs to ovigerous setae on the pleopods by a stalk. Often a large percentage of the clutch is lost during the 6–12 month brooding interval, especially in laboratory-maintained females. The factors responsible for egg loss during brooding are undefined. We have compared the morphological characteristics of egg stalks from wild and laboratory-spawned females and correlated these characteristics with egg retention. Our data show that the morphology of egg stalks varies among laboratory-maintained lobsters and that there is a strong positive correlation between abnormal stalk morphology and clutch attrition. We conclude that improper formation of the egg stalk is a major cause of egg loss in laboratory-maintained lobsters.

INTRODUCTION

During spawning, female lobsters (*Homarus*) position themselves on their backs and extrude eggs through the gonopores located at the base of the third walking legs (Herrick, 1909). The eggs pass posteriorly to the abdomen where they attach to the ovigerous setae of the pleopods or to other eggs by means of a stalk (funiculus). The mechanism of egg attachment is controversial and incompletely understood (Aiken *et al.* 1980; Fisher and Clark, 1983; Goudeau and LaChaise, 1983). In *Homarus*, the stalk is continuous with the outer coat surrounding the egg (Harper and Talbot, 1983). The eggs are brooded and develop on the pleopods for 6–12 months before hatching. In wild populations, *H. americanus* females may lose up to 36% of a clutch before hatching occurs (Perkins, 1971). Clutch attrition in laboratory-maintained females is often much greater than this; some females drop all of their eggs before hatching (Talbot *et al.*, 1984).

There are probably numerous factors that account for egg loss, although few have actually been investigated. Extrinsic factors include predation on egg masses by a nemertean (Aiken *et al.*, 1980) and removal and/or eating of eggs by the female (Knight, 1918). Epibiotic bacteria are present on the surface of the egg coat and stalk of both wild caught and laboratory-maintained *Homarus* (Harper and Talbot, 1984). There is not a clear correlation between density of epibionts and egg loss from clutches, although excessively high bacterial loads may affect retention and/or embryonic development. Faulty attachment of the eggs is a factor which could result in clutch attrition, as the quality of the stalk would seem critical in proper retention over the long brooding period. Our purpose in this study was to examine the morphology of egg stalks from wild-spawned and laboratory-spawned females and to correlate structural characteristics of the stalk with egg retention. We show that stalk morphology varies among females and that females having egg stalks morphologically different from wild spawned females do not retain their clutches well.

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MATERIALS AND METHODS

Animals

Egg samples from 74 berried female lobsters (*Homarus americanus* and *H. gammarus*) were obtained from sources reported previously (Harper and Talbot, 1984). The females studied were of the following 5 types: (1) *H. americanus,* born in the wild and spawned in the wild (n = 9), (2) *H. gammarus,* born in the wild and spawned in the laboratory (n = 16), (3) *H. americanus,* born in the wild and spawned in the laboratory (n = 24), (4) *H. americanus,* born in the laboratory and spawned in the laboratory (n = 11), and (5) hybrids of *H. gammarus* ♀ x *H. americanus* ♂ and the reciprocal cross, which were both born and spawned in the laboratory (n = 14). Females in group 1 were considered controls, *i.e.,* they had spawned in the wild, and their eggs were presumed to be attached to the pleopods normally.

Processing of egg samples

Most samples were taken from the periphery of the clutch near the middle of the abdomen. Eggs were fixed in glutaraldehyde as described previously (Harper and Talbot, 1984). They were rinsed in cacodylate and either stored at 4°C in cacodylate buffer (pH 7.4, 0.1 *M*), or they were dehydrated in a graded acetone series and stored at 4°C in 100% acetone.

Relative abundance, density, and morphological characteristics of stalk material

Egg stalks were examined with a dissecting microscope to determine if morphological differences in stalk quality existed among females in the five groups. After preliminary examinations, several descriptive terms were found useful in evaluating these stalks. The relative amount of stalk material was characterized as: (1) abundant: eggs were interconnected by large amounts of stalk material, (2) moderate: a significant amount of stalk material was present, but less than in the abundant category, and (3) sparse: very little stalk material was present (see Figs. 1–3 for examples).

The density of the stalk material was characterized by its ability to transmit light. Samples were ranked as: (1) opaque, (2) translucent, or (3) transparent. These evaluations were made on flat regions of the stalk, usually close to its attachment to the egg.

The stalks were also evaluated for the following morphological characteristics: (1) twisted: a portion of most stalks was twisted, (2) flat: large, flat expanses of stalk material were frequently seen, (3) broad: the interface between the egg coat and stalk was large, (4) wispy: the stalk had a delicate, fragile appearance, and (5) thin: the stalk was unusually narrow in diameter.

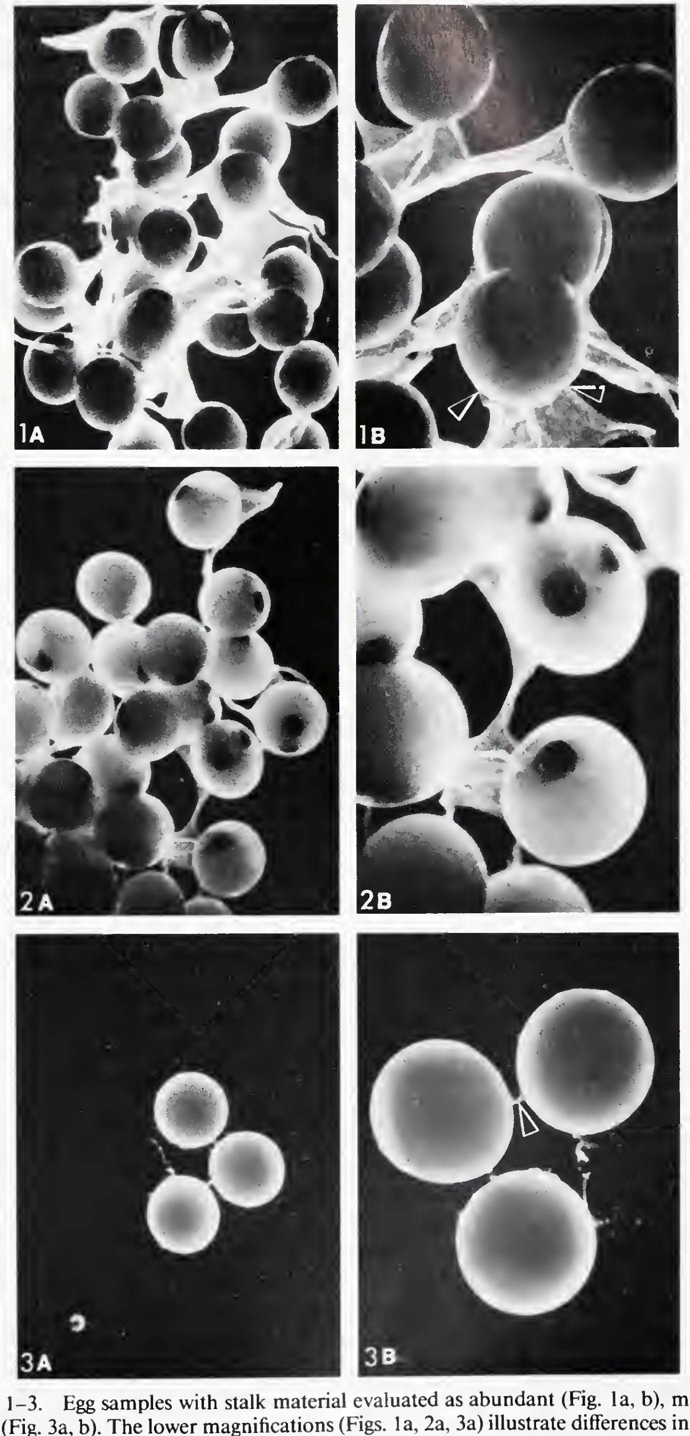
Egg retention

Records were kept at the laboratories of origin on egg retention for each female. These records were used to correlate egg retention with the observed morphological characteristics of the stalks for each female. All morphological data were collected before retention data were received making this aspect of the study blind. For some females, the number of eggs in clutches was estimated as described previously (Talbot *et al.,* 1984).

RESULTS

Relative abundance of stalk material

The amount of stalk material in each egg sample was rated as abundant, moderate, or sparse (Fig. 4). The wild born/wild spawned *H. americanus* and wild born/lab



FIGURES 1–3. Egg samples with stalk material evaluated as abundant (Fig. 1a, b), moderate (Fig. 2a, b), and sparse (Fig. 3a, b). The lower magnifications (Figs. 1a, 2a, 3a) illustrate differences in relative amount of stalk material. The higher magnifications (Figs. 1b, 2b) illustrate regions of stalks which were twisted, flat, and broad (attachment interface between arrows in Fig. 1b). The stalks in Figure 3b were evaluated as thin and wispy. Stalks in Figures 1b and 2b were evaluated as translucent.

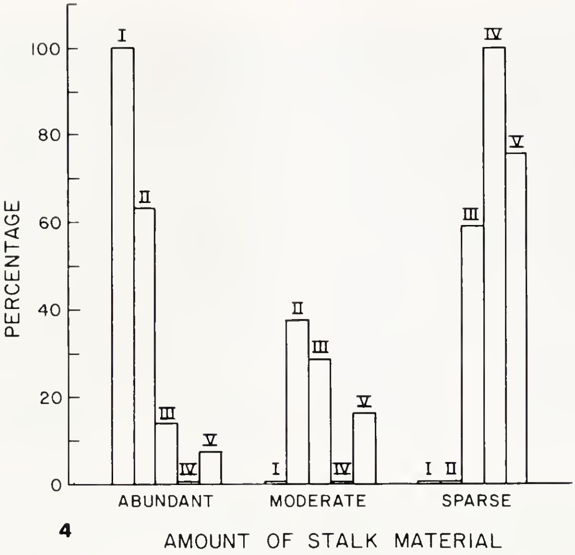


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FIGURE 4. The percentage of females in each of the 5 groups having stalks evaluated as abundant, moderate, or sparse. I = wild born/wild spawned *H. americanus;* II = wild born/lab spawned *H. gammarus;* III = wild born/lab spawned *H. americanus;* IV = lab born/lab spawned *H. americanus;* V = hybrids of *H. gammarus* × *H. americanus.*

spawned *H. gammarus* females all had abundant or moderate ratings. In contrast, most females in both groups of lab spawned *H. americanus* and the hybrid females had sparse amounts of stalk material.

Stalk density

The density of the stalk, as estimated by its opacity to light, is compared in Figure 5 for the 5 groups of females. Most wild born/wild spawned *H. americanus* and wild born/lab spawned *H. gammarus* had translucent stalks. However most stalk samples from females in the other 3 categories were rated transparent, suggesting they were thinner.

Morphological characteristics of the stalk material

The morphology of the stalks was evaluated for each egg sample using the descriptive terms twisted, flat, broad, thin, and wispy (Fig. 6). In almost all samples, some stalks were twisted. A high percentage of wild born/wild spawned *H. americanus* and wild born/lab spawned *H. gammarus* had stalks classified as broad and flat. In contrast, females in the other 3 groups most often had stalks which were thin and wispy.

Egg retention and quality of stalks

We have compared the relative abundance of the stalk material to egg retention times for each group of laboratory-maintained female (Table I). *H. americanus* females which were wild born/lab spawned showed variable results. Over 50% of these females had stalks evaluated as moderate or abundant. Egg loss did occur from these clutches,

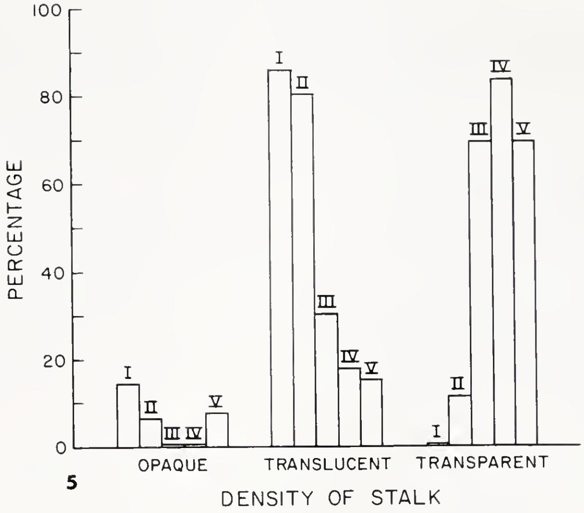

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FIGURE 5. The percentage of females in each of the 5 groups having stalks evaluated as opaque, translucent, or transparent. I = wild born/wild spawned *H. americanus;* II = wild born/lab spawned *H. gammarus;* III = wild born/lab spawned *H. americanus;* IV = lab born/lab spawned *H. americanus;* V = hybrids of *H. gammarus* × *H. americanus.*

and with the exception of one female (645;BML), loss was gradual over time. Eighty percent of the females carried at least a portion of their clutch through to hatching. Within the same group, 44% had stalks evaluated as sparse, and all except B-136 lost their eggs rapidly.

H. americanus females which were lab born/lab spawned all had sparse stalks (Table I), except for one female (R-130) which was evaluated as sparse/moderate.

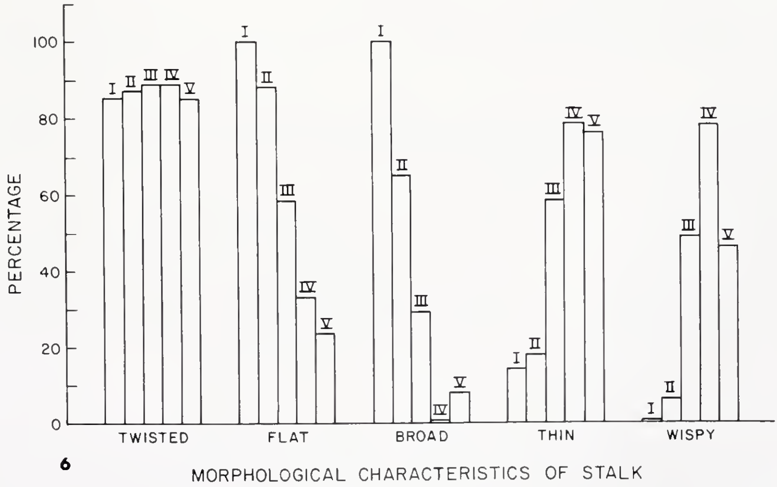

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FIGURE 6. The percentage of females in each of the 5 groups having stalks evaluated as twisted, flat, broad, thin, or wispy. I = wild born/wild spawned *H. americanus;* II = wild born/lab spawned *H. gammarus;* III = wild born/lab spawned *H. americanus;* IV = lab born/lab spawned *H. americanus;* V = hybrids of *H. gammarus* × *H. americanus.*

TABLE I

Amount of stalk material and egg retention

Stalk evaluation	% Attaching large clutches	% Gradual loss	% Rapid loss	% Hatching[1]
Wild Born/Lab Spawned *H. americanus* (n = 18)				
number abundant = 5 number moderate = 5	—	90%	10%	80%
number sparse = 8	25%	12.5%	87.5%	12.5%
Lab Born/Lab Spawned *H. americanus* (n = 6)				
number abundant or moderate = 0	—	—	—	—
number sparse = 6	16.7%	16.7%	83%	0%
Hybrids (n = 9)				
number abundant = 1	—	—	100% (eggs unfertilized)	0%
number sparse = 8	66.6% (n = 6)	0%	100%	0%
Wild Born/Lab Spawned *H. gammarus* (n = 13)				
number moderate or abundant = 13	100% (n = 7)	85%	15%	77%
number sparse = 0	—	—	—	—

[1] "Hatching" indicates that some eggs in the clutch were carried through to hatch.

All females, except R-130, attached 500 or fewer eggs, and these were lost rapidly. Female R-130 attached a large clutch (14,200) and lost all her eggs gradually before hatching.

Of the 9 hybrids, all except female 4011 had sparse stalks. Although most (67%) females attached a large clutch, eggs were lost rapidly and none were carried through to hatching. Female 4011 attached a small clutch of unfertilized eggs (she had not been mated) and lost these rapidly.

All *H. gammarus* females (n = 13) had moderate/abundant stalk evaluations. Initial clutch size data were available for 7 females, and all of these attached large clutches. Eleven of the 13 experienced gradual attrition; 1 of 13 lost her eggs rapidly and no data on attrition were available for the other female. Of the 13 females studied, 10 carried at least a portion of their clutch through to hatching.

Effect of fertilization on stalk morphology

Egg samples from 70 females were examined for evidence of fertilization. Eggs were considered fertilized if normal cleavage patterns or eye spot stages were observed. All wild born/wild spawned females had fertilized eggs and abundant stalks. For laboratory-maintained females, 49 of 63 females had fertilized eggs; 10 of 63 had eggs which could not be categorized unequivocally; and 4 of 63 females had eggs which were not fertilized. For the 4 unfertilized samples, stalks were categorized as abundant in 2 cases, moderate in one case, and undetermined in one case. Moreover, all hybrid females and all lab born/lab spawned females which had fertilized eggs produced stalks rated as "sparse." We conclude that production of "sparse" stalks was not caused by failure of the eggs to be fertilized.

DISCUSSION

We have examined morphological characteristics of egg stalks from wild- and laboratory-maintained lobsters (*Homarus*) and correlated these characteristics with egg retention. Our data show that the morphology of the egg stalk varies among different groups of laboratory-maintained females. For control females (wild born/wild spawned *H. americanus*), stalks were most often evaluated as abundant, translucent, twisted, flat, and broad. Females having stalks of this type generally carry many eggs through to hatching, suggesting these are morphologically advantageous characteristics.

Laboratory-maintained females showed variations in the morphology of their egg stalks. All *H. gammarus* and about half the wild born/lab spawned *H. americanus* had stalks resembling those of the control group and with a few exceptions, such females carried some eggs through to hatching. This indicates that laboratory-maintained females producing stalks of the wild type are able to carry their clutches over the long brooding interval and supports the idea that stalk morphology affects egg retention. However, even in these clutches, attrition occurred. In some, but not all, instances, it was probably no greater than would be expected in a wild population (36%, Perkins, 1971). Thus, while good stalks are essential in clutch retention, even females with good stalks may experience considerable egg loss. The factors contributing to loss in such cases are probably subtle and should be subjected to further investigation.

The majority of laboratory-maintained females had stalks that were morphologically different than those of the controls. Females with stalks ranked as sparse included about half the wild born/lab spawned *H. americanus,* all the lab born/lab spawned *H. americanus,* and all the hybrids. These females often attached small clutches which they lost rapidly (both groups of *H. americanus*) or attached large clutches which they lost rapidly (hybrids). We conclude that much of the egg loss observed in laboratory-maintained females (Talbot *et al.,* 1984) can be attributed to the production of morphologically abnormal stalks.

Egg retention in captive, and perhaps in wild, females could be improved by more consistent production of wild-type egg stalks. The origin of the egg stalk has been the subject of controversy for years (see Fisher and Clark, 1983; Goudeau and La Chaise, 1983). In *Carcinus* (Goudeau and LaChaise, 1983) and in the lobster *Jasus* (Silberbauer, 1971) the egg stalk forms from an investment laid down while the egg is in the ovary. In *Homarus,* the egg stalk is formed from the chorion, a coat present around ovarian oocytes and comparable to the vitelline envelope of *Carcinus* (Talbot, 1983). The "sparse" stalks observed in this study may have resulted from incomplete or faulty synthesis of the chorion by some laboratory-maintained females. The chorion in *Homarus* is produced by follicle cells (Talbot, 1981), but the factors which regulate chorion synthesis are not known. Laboratory conditions in this study did not simulate wild conditions, and female reproductive physiology may be erratic in the higher ambient water temperatures found in some laboratories. Waddy and Aiken (1983) have shown that a winter water temperature of less than 10°C increases the percentage of females extruding eggs. It is also possible that environmental factors such as water temperature indirectly affect completion of chorion synthesis. This could be tested by maintaining laboratory females on a photoperiod and at a water temperature comparable to that of their native environment. Other factors, such as an incomplete hardening of the chorion after fertilization, may also influence proper formation and functioning of the egg stalk.

We conclude that the production of abnormal egg stalks is a major, but not the only, factor contributing to clutch attrition in laboratory-maintained lobsters and that the prevention of egg loss will be attained through a better understanding and control of chorion synthesis and formation of the egg stalk.

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MODIFICATION OF TUBULIN WITH THE FLUOROCHROME 5-(4,6-DICHLOROTRIAZIN-2-YL)AMINO FLUORESCEIN AND THE INTERACTION OF THE FLUORESCENT PROTEIN WITH THE ISOLATED MEIOTIC APPARATUS

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ABSTRACT

Microtubules (MTs) assembled *in vitro* have been labeled with the fluorochrome 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF) using a modification of the procedure of Keith *et al.* (1981). When the fluorescent MTs were analyzed by polyacrylamide gel electrophoresis under reducing conditions both the major components of the MTs, the tubulin dimers and microtubule-associated proteins (MAPs), appeared distinctly fluorescent and thus were modified covalently with the fluorochrome. The fluorescent MTs were cold labile and reassembled with kinetics identical to control microtubule proteins. In addition, fluorescent tubulin dimers, purified from the mixture of labeled MT proteins, assembled with both an initial rate and final extent of assembly indistinguishable from a control, unlabeled sample when both were examined at the same protein concentration. On occasion, however, the fluorescent protein has been observed to demonstrate a one to two minute lag, an anomaly that is a function of total protein concentration. When the reassembled fluorescent MTs were examined by negative stain electron microscopy, morphologically normal polymers were observed. Since fluorescent tubulin dimers are potentially valuable probes for the examination of MT growth polarity, the ability of the fluorescent protein to assemble in association with nucleation centers *in vitro* was examined. For these experiments, meiotic spindles from the surf clam *Spisula solidissima* were isolated and used to nucleate the assembly of fluorescent MTs *in vitro*. The results of such experiments revealed linear, fluorescent arrays within the spindle and asters. The results of the experiments reported here demonstrate that tubulin labeled with DTAF retained its ability to self-assemble and to assemble in association with microtubule organizing centers (MTOCs) *in vitro*. Moreover, the polymer formed from the DTAF labeled proteins was distinctly fluorescent and could be observed and recorded using conventional fluorescence optics.

INTRODUCTION

Much of our knowledge of the cytoskeleton has been obtained from conventional electron microscopy and immunocytochemical techniques. Ultrastructural analyses (Porter, 1966; Tilney, 1971; Willingham *et al.,* 1981) revealed details of the structural organization of the cytoplasm, while immunocytochemistry, performed using antibodies specific for particular cytoskeletal proteins, illustrated the distributions of, and spatial relationships among, these components (Lazarides, 1980; Ball and Singer,

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1981; Herman *et al.*, 1981). However, because these techniques require fixed preparations, rearrangements of cytoskeletal proteins during motile events cannot be determined directly.

To investigate the molecular dynamics of the cytoskeleton in living cells, Taylor and Wang (1978) introduced the technique of Molecular Cytochemistry, more recently termed Fluorescent Analog Cytochemistry (FAC, Wang *et al.*, 1982). In this technique, based on the initial work of Sanger (1975), fluorescent derivatives of cellular proteins which retain their native characteristics were prepared and introduced into living cells; the cells were then observed with a fluorescence microscope. This technique, which has been used to study a variety of different cytoskeletal components (Taylor and Wang, 1978; Feramisco, 1979; Kreis *et al.*, 1979; Burridge and Feramisco, 1980; Gawlitta *et al.*, 1980; Keith *et al.*, 1981; Glacy, 1983), has revealed the incorporation of the fluorescent derivatives into cellular structures. More importantly, the distribution and the changing pattern of the fluorescent protein can be monitored in living cells during various motile events. This report describes a modification of the procedure of Keith *et al.* (1981) for the preparation of tubulin modified with the fluorochrome 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF). The procedure is rapid and yields modified, fluorescent tubulin in quantitatively reproducible amounts, a result not achieved using previously published procedures. The modified protein probe retains its native characteristics as judged by a number of criteria; moreover the fluorescent derivative incorporated into MTs of isolated spindles which could be observed using conventional fluorescence microscopy.

Materials and Methods

Protein purification

Microtubule proteins (MTP) were purified from pig brains using cycles of temperature-dependent assembly and disassembly as described by Sloboda *et al.* (1976). Polymerization buffer (PM) consisting of 0.1 *M* Pipes, pH 6.9; 1 m*M* $MgSO_4$; 2 m*M* EGTA; and 1 m*M* GTP was used throughout the procedure. Brain tissue was homogenized using a glass teflon homogenizer with a motor driven pestle in 0.5 ml of buffer per gram of tissue. The supernatant obtained after centrifugation of the brain homogenate for 1 hour at 100,000 × *g* was mixed with an equal volume of PM buffer containing 8 *M* glycerol (PMG). MTs, assembled during incubation at 37°C for 40 min, were collected by centrifugation at 100,000 × *g* for 40 min, resuspended in fresh PM using a dounce homogenizer, and depolymerized on ice for 30 minutes. The depolymerized MTs were clarified by centrifugation at 100,000 × *g* for 30 min. The supernatant was again mixed with an equal volume of PMG, polymerized, and spun as before. The sedimented MTs were overlaid with PM containing 4 *M* glycerol and stored at −70°C until use. Tubulin dimers were prepared free of MAPs using phosphocellulose column chromatography (Weingarten *et al.*, 1975; Williams and Detrich, 1979; Sloboda and Rosenbaum, 1982). Column buffer consisting of 50 m*M* Pipes, pH 6.9; 0.5 m*M* $MgSO_4$; 1.0 m*M* EGTA; and 0.1 m*M* GTP was used throughout the procedure.

When required, fluorescent MAPs were prepared using a modification of the taxol procedure of Vallee (1982). Briefly, three times cycled MTs were polymerized in PM buffer containing 20 μM taxol (PMT). The MTs were then labeled with the fluorochrome DTAF for 5 min at 37°C (see Results) and collected by centrifugation at 100,000 × *g* for 30 min. The fluorescent MTs were resuspended and washed once in PMT. The salt concentration of the resuspended MTs was then adjusted to 0.35 *M* using a concentrated solution of NaCl. The fluorescent MTs, which had been stripped of MAPs by the salt, were separated from the solubilized MAPs by centrifugation at 30,000 × *g* for 30 min. The fluorescent MAPs were recovered from the supernatant and desalted on Sephadex G-25 using centrifuge desalting columns (Neal and Florini, 1973).

Electron microscopy

Diluted solutions of microtubules were applied to formvar coated copper grids, allowed to adsorb for approximately 1 min, and stained with millipore filtered aqueous uranyl acetate and air dried. Grids were examined in a JEOL 100CX electron microscope operated at 60kV.

Meiotic spindle isolation

Meiotic spindles were isolated from activated *Spisula solidissima* oocytes as described by Murphy (1980) with several minor changes. Ooctyes, activated by raising the potassium concentration of the sea water by 38 m*M* using a 0.52 *M* KCl solution (Allen, 1954), were sedimented in a hand centrifuge, washed twice in 1 *M* glycerol, 1 m*M* Tris, pH 8.0, and 2 m*M* EGTA, and resuspended in Isolation Medium (IM) consisting of 10 m*M* MES, pH 6.2; 5 m*M* EGTA; 1.0% Nonidet NP-40; 0.5 *M* glycerol, and 0.5 m*M* $MgSO_4$ at room temperature. The eggs were lysed by shaking and the isolated meiotic spindles were collected by centrifugation for 5 min at 3000 × *g* in a clinical centrifuge.

Microscopy

Phase contrast, fluorescence, and polarized light microscopy were performed using a Zeiss Photomicroscope III available through the generous loan of the Carl Zeiss Co. to the students and staff of the Physiology Course, Marine Biological Laboratory, Woods Hole, Massachusetts. For phase contrast and fluorescence observations, a Zeiss 63× planapochromat lens (N.A. = 1.4) was used. A 10× pol objective and Brace-Kohler compensator were used for polarized light observations.

Other methods

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using a 5–20% gradient of acrylamide. Proteins were stained with Coomassie Brilliant Blue R according to Fairbanks *et al.*, (1971). Unstained gels were fixed in 10% acetic acid, washed in 50 m*M* Tris, 150 m*M* NaCl, 0.02% NaN_3, pH 7.5 (Burridge, 1976), and photographed under long wave-length ultraviolet illumination using an Edna Lite 606 filter and Tri-X film. Turbidity measurements (Gaskin *et al.*, 1974) were performed using a Gilford model 250 recording spectrophotometer fitted with a thermal cuvette holder. Fluorescence measurements were made using a Turner model 430 spectrofluorometer equipped with an Aminco photon counter and a 150 watt Xenon lamp. Protein concentration was determined by the method of Lowry *et al.* (1951) as modified by Schacterle and Pollack (1973) using a BSA standard.

RESULTS

Fluorescent labeling of microtubules

MTs in the assembled state were labeled with fluorochrome so that sites on the tubulin dimers critical for the polymerization of a MT would be protected from reaction with the fluorochrome. The fluorescent labeling reagent 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF), which reacts covalently with ϵ-amino groups of lysine residues and N-terminal amino groups (Blakeslee and Baines, 1976), was mixed with the polymerized MTs at 37°C. No decrease in turbidity, which would indicate MT disassembly, was observed during the labeling reaction. The fluorochrome, prepared fresh daily in dimethyl-sulfoxide, and assembled microtubule proteins were combined using a 2–4 fold molar excess of fluorochrome to protein; the labeling reaction was performed for 5 min (see Keith *et al.*, 1981). Immediately after labeling centrifugation was begun to collect the fluorescent polymer and separate the MTs from the bulk of the fluorochrome, thereby terminating the labeling reaction. The fluorescent MT pellets were resuspended in fresh PM and depolymerized on ice. To ensure that any remaining unbound fluorochrome was removed from solution, the depolymerized proteins were desalted using Sephadex G-25 in centrifuge columns (Neal and Florini, 1973). The fluorescent proteins were then subjected to a further round of temperature-dependent assembly and disassembly to select for assembly competent proteins. Purified fluorescent tubulin dimers were prepared using phosphocellulose column chromatography (see Methods). Thus, the major modifications of the technique of Keith *et al.* (1981) reported here are (i) a decrease in fluorochrome to protein ratio during labeling, (ii) a decrease in labeling time, and the addition of (iii) a centrifugation step, (iv) a gel chromatography step, and (v) a further round of assembly and disassembly prior to purification of the labeled dimers by phosphocellulose chromatography.

Characteristics of the fluorescent proteins

When the fluorescent MT proteins were analyzed by polyacrylamide gel electrophoresis both the α- and β-tubulins and high molecular weight microtubule-associated proteins (MAPs, Sloboda *et al.*, 1975) were distinctly fluorescent. In addition, after purification of the tubulin dimers by phosphocellulose chromatography and the MAPs by a taxol procedure (see Methods), both protein fractions remained distinctly fluorescent (Fig. 1); the fluorescence co-migrated with the protein and no fluorescent breakdown products were observed. Since these are denaturing gels run under reducing conditions (see Methods), these observations demonstrate that the fluorochrome is covalently bound to the protein.

Several experiments were performed to determine if the modified proteins retained their native characteristics. For example, the assembly kinetics of the fluorescent proteins were compared with control unlabeled proteins assembled at the same protein concentration. As seen in Figure 2, three times cycled, fluorescent microtubule proteins assembled with both an initial rate and final extent of assembly identical to the unlabeled proteins. Next, purified fluorescent tubulin dimers were prepared and tested to determine if they also retained the same assembly characteristics as unlabeled tubulin dimers after separation from the MAPs. As seen in Figure 3, the fluorescent tubulin dimers assembled with kinetics indistinguishable from the unlabeled control sample. The lag apparent in the experimental sample (F) in Figure 3 occurs occasionally at low protein concentrations (1–3 mg/ml). When examined using negative stain electron microscopy, MTs assembled from the fluorescent subunits were observed to be morphologically normal (Fig. 4).

In addition, the excitation and emission spectra of the fluorescent microtubule proteins were determined. The excitation and emission maxima were 495 nm and 520 nm, respectively (Fig. 5). The wavelengths of maximal excitation and maximal emission were identical using either polymerized or depolymerized MT proteins for the determination. These data suggest that polymerization does not alter these values within the resolution of the instrument used.

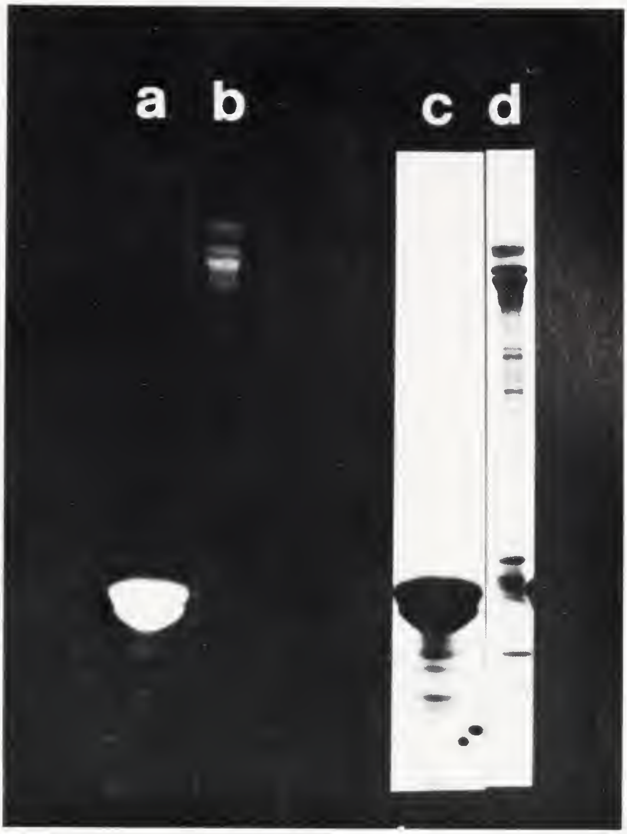

FIGURE 1. Polyacrylamide gel electrophoresis of the fluorescently modified proteins. The unstained gels were photographed under UV illumination to visualize the fluorescence (a and b); identical gels were then stained for protein with Coomassie Brilliant Blue (c and d). Lanes a and c are phosphocellulose purified DTAF labeled tubulin dimers; lanes b and d are DTAF labeled MAPs prepared according to Vallee (1982) (see Methods). Both the α- and β-subunits of tubulin are distinctly fluorescent as are the high molecular weight MAPs.

The stoichiometry of labeling was determined as described by Keith *et al.,* (1981) using an extinction coefficient of 5.4×10^5 for DTAF and a molecular weight of 110,000 for the tubulin dimer. At a final stoichiometry of 0.003–0.1 (moles of fluorochrome bound per mole of protein) the proteins retained their native characteristics and were distinctly fluorescent.

Interaction of the fluorescent protein with the isolated mitotic apparatus

To determine whether the fluorescent protein retained the ability to assemble in association with microtubule organizing centers (MTOCs, Pickett-Heaps, 1969) *in vitro,* the fluorescently modified protein was incubated with meiotic spindles isolated from oocytes of the surf clam, *Spisula solidissima.* Incubation of isolated spindles with fluorescent MT protein maintained or augmented the birefringence of the isolated spindles in a temperature and concentration dependent fashion (Fig. 6). For example, when isolated *Spisula* meiotic apparatuses (MAs) (Fig. 6a) were resuspended in po-

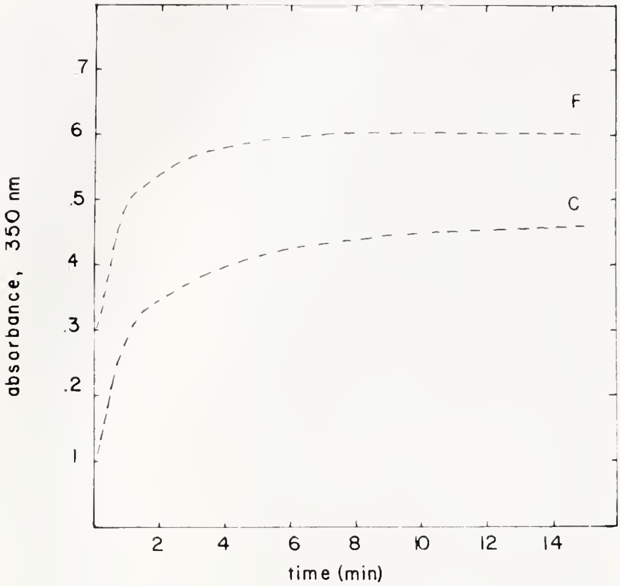

FIGURE 2. Kinetics of assembly of a DTAF labeled (F) and control (C) sample of microtubule proteins, each at 2.0 mg/ml. Assembly was monitored at 37°C using a temperature controlled cuvette holder. Apparent absorbance at 350 nm has been plotted as a function of time; the initial A_{350} values have been offset along the ordinate using the controls on the spectrophotometer. Note that the total change in A_{350}, indicative of the total mass of assembled polymer, is the same for both samples, as is the initial rate of assembly.

lymerization buffer (PM) and placed on ice, the spindle MTs disassembled and only very faint birefringence (BR) remained (Fig. 6b). However, when aliquots of the same preparation of spindles (Fig. 6a) were resuspended in PM buffer containing MT proteins, chilled, and rewarmed, spindle BR was observed in proportion to the concentration of MT proteins present (Fig. 6c, d).

When the isolated spindles, which had been resuspended in solutions containing fluorescent microtubule proteins, were examined using fluorescence optics, distinctly fluorescent spindles with linear, fluorescent fibers emanating from the spindle poles were seen (Fig. 7a, b). This result suggests that the fluorescent proteins were incorporated into the spindle and astral regions of the MA, an observation consistent with previous experiments performed using unlabeled brain MT proteins to augment the BR of meiotic (Inoué *et al.*, 1974; Rebhun *et al.*, 1974) or mitotic (Cande *et al.*, 1974) spindles. When calcium was added to the fluorescent spindles, a rapid change in their appearance was observed (Fig. 7c, d). The linear fluorescent fibers were reduced to amorphous fluorescence (Fig. 7d). Similarly, the linear appearance of the spindle fibers observed in phase contrast was abolished (Fig. 7c). This sensitivity of the fluorescence to millimolar concentrations of calcium suggests that the fluorescent pattern shown in Figure 7b was due to the polymerization of calcium labile, fluorescent MTs.

DISCUSSION

The results presented here demonstrate that MTs can be modified with the fluorochrome DTAF as previously reported in Keith *et al.* (1981). The modification of the microtubule proteins with the fluorochrome is covalent, as demonstrated in Figure

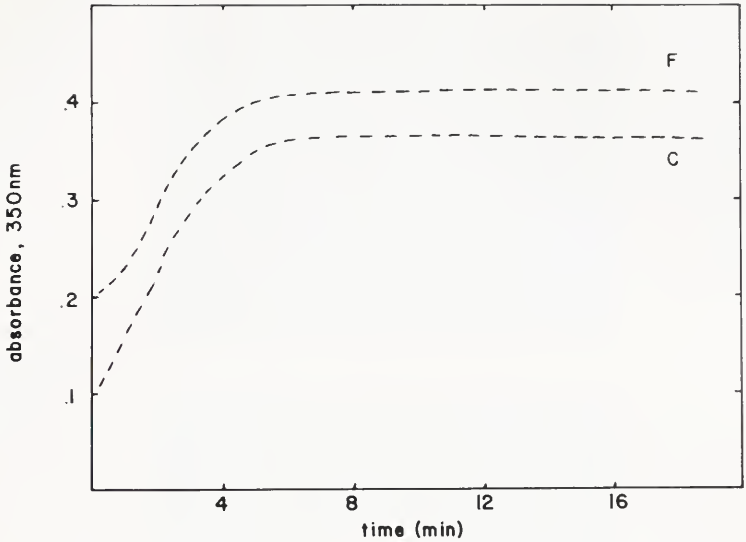

FIGURE 3. Kinetics of assembly of a control (C) and DTAF labeled (F) sample of phosphocellulose purified tubulin dimers, each at 3.2 mg/ml. For this experiment, the fluorescent protein was prepared using a 2:1 molar excess of fluorochrome to protein. Assembly was monitored at 37°C using a temperature controlled cuvette holder. Apparent absorbance at 350 nm has been plotted as a function of time; the initial A_{350} values have been offset along the ordinate using the controls on the spectrophotometer. As in Figure 2, the total change in A_{350} is nearly identical for each sample, as is the initial rate of assembly.

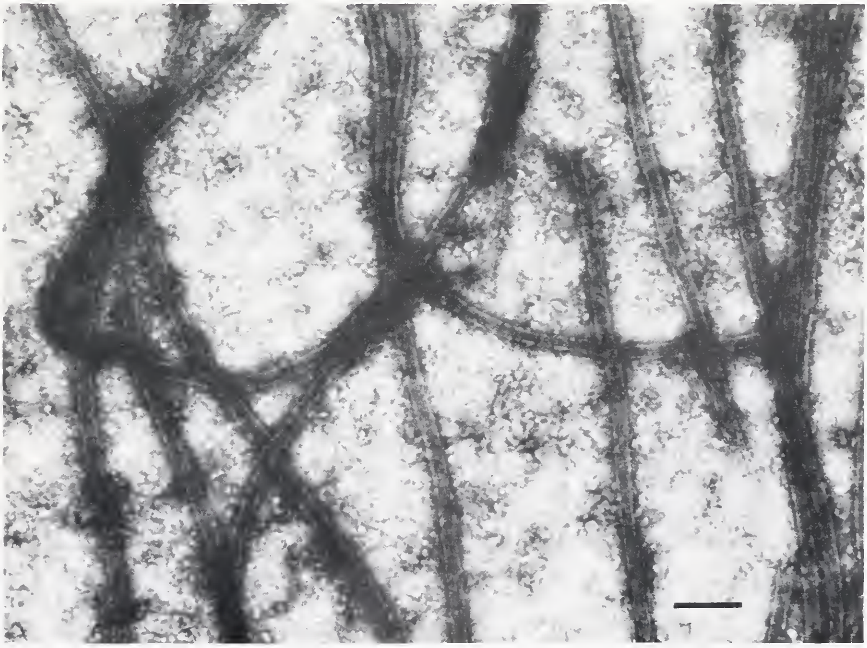

FIGURE 4. Negative stain electron micrograph of assembled fluorescent microtubules. The MTs appear morphologically normal. Bar = 100 nm.

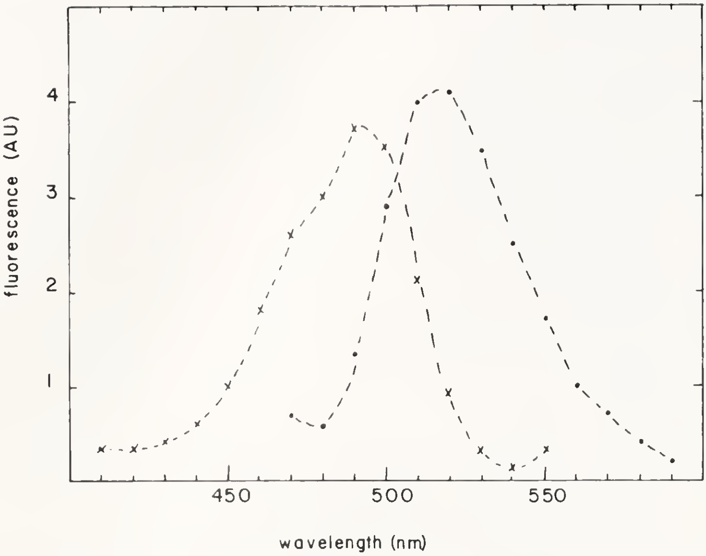

FIGURE 5. Excitation/emission spectra of DTAF labeled microtubule proteins. The excitation maximum (--×--×--) is 495 nm and the emission maximum (--●--●--) is 520 nm. These values are the same whether intact MTs or depolymerized MT proteins are used for the determination. AU = arbitrary units.

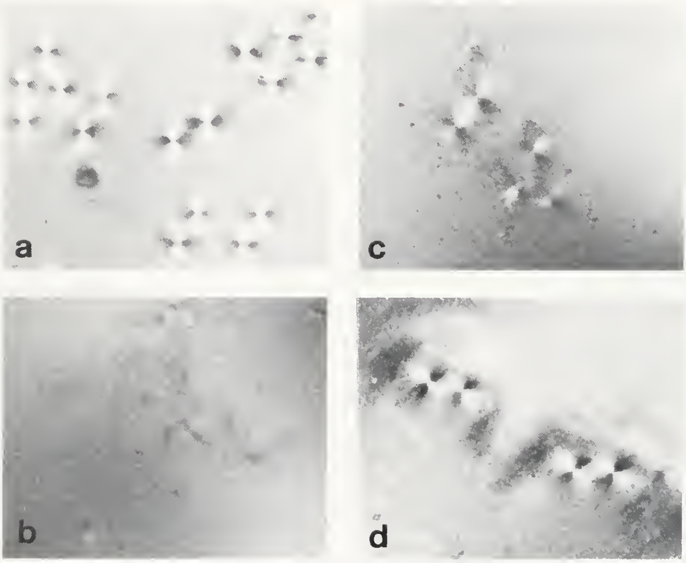

FIGURE 6. Polarized light micrographs demonstrating the effect of fluorescent microtubule proteins on the birefringence of isolated meiotic spindles from the surf clam *Spisula solidissima.* (a) Polarized light micrograph of the spindles immediately after isolation. (b) The same preparation of spindles after centrifugation and resuspension in PM buffer at 4°C. When resuspended in PM buffer containing (c) 0.8 mg/ml and (d) 3.8 mg/ml fluorescent microtubule proteins, followed by incubation at 37°C, BR is observed in proportion to the concentration of fluorescent proteins present.

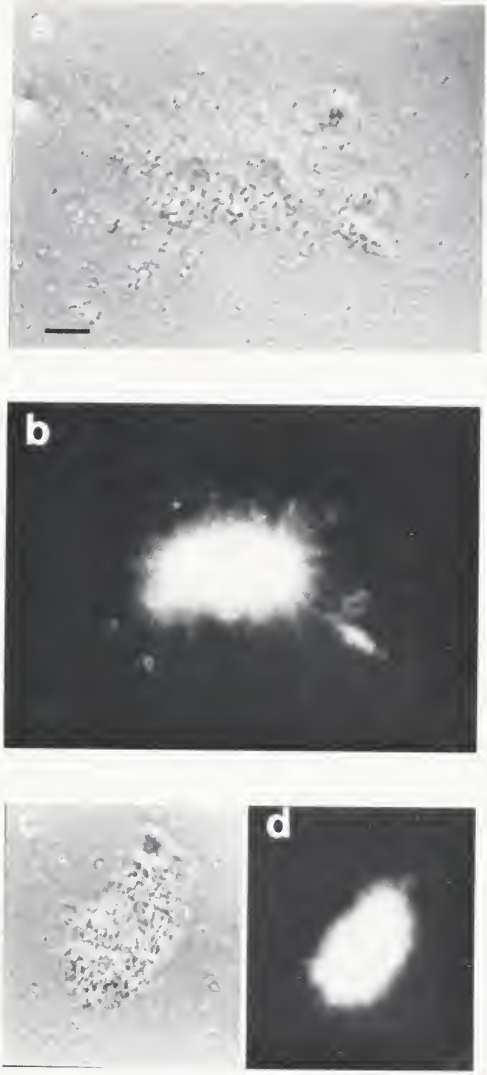

FIGURE 7. Phase contrast (a, c) and fluorescence (b, d) micrographs of *Spisula solidissima* meiotic spindles. (a, b) The spindles were incubated at 37°C with DTAF labeled microtubule proteins at 3.8 mg/ml. Note the distinct, linear fluorescence. (c, d) 3×10^{-3} *M* calcium has been added to the fluorescent spindles; note the loss of linear fluorescence (d) and the alteration in spindle morphology in phase contrast (c). Bar = 20 μm.

1. Moreover, the fluorescent proteins retain their native characteristics with respect to several criteria. For example, the modified proteins assemble with kinetics indistinguishable from controls and appear morphologically normal when examined in negatively stained preparations (see Figs. 2–4). In addition, the fluorescent microtubule protein is capable of assembly in association with isolated spindles *in vitro* and can be visualized and photographed using conventional fluorescence microscopy (Fig. 7).

The resulting stoichiometry for fluorescent tubulin reported here (*ca.* 0.1 mole/mole tubulin) is less than the 0.5 mole fluorochrome: mole tubulin dimer previously reported for tubulin modified with DTAF (Keith *et al.*, 1981). Preliminary experiments, performed using the labeling conditions described by Keith *et al.* (1981), resulted in very low yields of protein that had poor assembly characteristics. Thus, modifications of the Keith *et al.* (1981) labeling procedure were made (see Results). This modified procedure yields tubulin which retains its native characteristics, forms distinctly fluorescent polymers (see Fig. 7), and is therefore suitable for use as a probe of MT assembly *in vitro* and *in vivo.* However, it should be pointed out that much of the tubulin is denatured after exposure to even the modified labeling conditions reported here; thus the DTAF labeled tubulin can only be prepared with a maximum yield of approximately 5.0%.

The labeling procedure described here is a convenient method for preparation of fluorochrome labeled tubulin and MAPs. The tubulin retains its native characteristics and is suitable for use in the technique of fluorescent analog cytochemistry (Wang *et al.*, 1982). As demonstrated by Keith *et al.* (1981), purified, DTAF-labeled tubulin dimers or DTAF-labeled MT proteins compete quantitatively with unlabeled proteins for assembly into polymer. While such an *in vitro* competition assay has not been repeated here, microinjection of DTAF labeled tubulin into living cells demonstrates that this modified tubulin competes successfully with a large excess of tubulin for assembly into polymer (Wadsworth and Sloboda, 1983). Experiments in progress also indicate that the DTAF labeled MAPs retain the ability to bind to MTs *in vivo,* and may be useful probes for analyzing MAP function in living cells.

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ENTOCLADIA ENDOZOICA SP. NOV., A PATHOGENIC CHLOROPHYTE: STRUCTURE, LIFE HISTORY, PHYSIOLOGY, AND EFFECT ON ITS CORAL HOST

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ABSTRACT

A new species of the marine microalgal genus *Entocladia* (Ulvellacea, Chlorophyta) is described from cultured material and from its natural habitat, the skeleton of a gorgonian coral (*Pseudoplexaura* spp.). Host tissues react to the presence of this filamentous chlorophyte by producing a capsule composed primarily of scleroprotein skeleton, and secondarily of calcareous spicules. The skeletal capsule separates the algae from contact with host tissue but in doing so the skeleton loses more than 60% of its tensile strength and over 90% of its elasticity. The coral colony readily breaks apart at the site of the weakened skeleton, exposing the contained algal filaments to relatively high light fields and sea water, an amino acid-depleted environment. These conditions lead to rapid cytological changes that convert the alga to a reproductive state. Regeneration of damaged host tissue re-seals the capsule and causes resumption of the vegetative condition typical of enclosed algae. Experiments with native and cultured material suggest that host-derived amino acids, especially tyrosine or cystine, play a key role in regulating the reproductive condition of *Entocladia* filaments.

INTRODUCTION

Algal pathogens have been associated with a wide variety of host taxa but have been noted only occasionally in the literature. A form of lymphadenitis in cattle and sheep is apparently caused by a green alga (Rogers *et al.*, 1980) while other chlorophytes attack higher plants (Joubert and Rijkenberg, 1971) or cause pathogenesis in certain bivalve molluscs (Naidu, 1971). Recently, Morse *et al.* (1977, 1981) described the occurrence of tumor-like growths associated with a siphonaceous green alga in sea fans of the genus *Gorgonia.* A different chlorophyte is associated with abnormal growths in gorgonian corals of the genus *Pseudoplexaura* particularly in the shallows of the Florida Keys (Fig. 1). Of 1377 colonies examined from the northern Florida Keys (Soldier Key) to the Dry Tortugas, one third bear nodules. In certain reef areas containing significant *Pseudoplexaura* populations, up to 54% of the colonies exhibit this pathology (Goldberg and Makemson, 1981). We have identified the pathogen as *Entocladia endozoica* sp. nov., a marine microalga belonging to the recently resurrected family Ulvellaceae (Ulvales, Chlorophyta) (O'Kelly and Yarish, 1980; O'Kelly, 1983). In this report we describe the alga as a species that is remarkable not only for its endozoic habitat, but also for its pathogenic effect on host skeleton formation, which in turn appears to control the alga's life history. Aspects of the host's cellular response are also considered.

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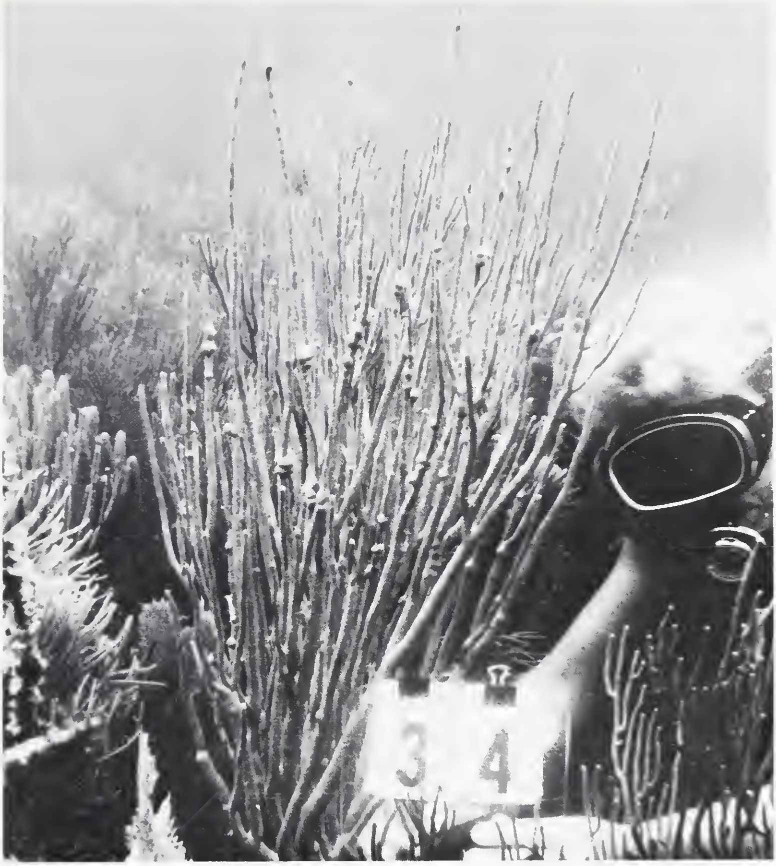

FIGURE 1. *Pseudoplexaura flagellosa* (Houttuyn) with algal nodules, depth 3 m, Bache Shoal, Florida Keys.

MATERIALS AND METHODS

Isolation and culture of the alga

Tumors were collected from several species of *Pseudoplexaura* in Florida and the Netherlands Antilles. Algal filaments were removed by forceps from surface sterilized (alcohol) tumors and washed repeatedly on Whatman filters with artificial sea water (Baumann *et al.,* 1971). Sterile technique was used in all procedures. The alga was isolated in pure culture by streaking agar plates containing Provasoli enrichments (McLachlan, 1973) added to artificial or natural sea water with the addition of 10 m*M* Tris buffer, pH 7.5. Cultures were grown at 25°C under a bank of cool white fluorescent lamps at 1000 lux on a 12:12 cycle. Within two weeks, dark green colonies (see Results, Fig. 4) became visible on the plates. The colonies were re-streaked on the same medium and on Difco-Marine Agar to check for purity and bacterial contamination. Stock cultures were maintained as slants or plates at 25°C (12:12) with bimonthly transfers. Morphological and growth studies required transfer of cells from the agar surface to a modified Provasoli's (liquid) medium, followed by gentle glass homogenization. Cell cultures (25 μl) were grown in Terasaki plates containing filter paper wicks moistened with 2% $NaHCO_3$. Physiological experiments employed similarly prepared cell suspensions incubated in tubes at 0, 100, or 1000 lux at 25°C. Cell numbers were determined by a hemacytometer, counting only chloroplast-containing cells. Pigments were analyzed from ice cold 90% acetone extracts in a Varian 635 double beam spectrophotometer and by cellulose thin layer chromatography using a methanol:acetone:water (30:10:3) solvent system (Kleinig, 1969; O'Kelly, 1982). Pigments were compared to those of the alga *Avrainvillea nigricans* Decaisne freshly collected from Key Largo, Florida.

Radiochemicals were from New England Nuclear (Boston, Massachusetts). Scintillation counting of radioactivity employed a Beckman 9000 liquid scintillation counter using Hydromix (Yorktown Research, Hackensack, New Jersey); a final concentration of 0.1 *M* hyamine was used in samples containing labeled carbon dioxide.

Histology, ultrastructure, and skeletal mechanics

Histological observations were made only on *Pseudoplexaura flagellosa* (Houttuyn) fixed in Bouin and stained with Gomori's trichrome after normal paraffin embedment. Tissue volumes were calculated from undehydrated, fixed cross sections of branches 1 mm thick. Spicular weight was determined after treatment with commercial bleach (5% NaOCl). Material for transmission electron microscopy was fixed 2–4 hours at 4°C in several primary fixative mixtures. Most frequently we used 2% glutaraldehyde and 4% paraformaldehyde in 0.1 *M* cacodylate buffer. Addition of sucrose brought the specific gravity of the mixture to sea water tonicity. Post-fixation employed 1% OsO_4 in the same buffer and temperature for 1 hour. Long dehydration times were required for proper infiltration and embedment in Polybed 812 (Polysciences, Warrington, Pennsylvania). In later phases of this study we found that embedment with Spurr resin (Polysciences) gave better results. Silver to gold sections were cut with a diamond knife, placed on uncoated copper grids, and photographed with a Philips 200 electron microscope.

Primary fixation for scanning electron microscopy was as above. Fixed algal swarmers from cultured material were allowed to settle for 15 minutes on Nucleopore filters (0.22 μm) that had been treated with a 0.1% aqueous solution of polylysine (Sigma, MW = 350,000). This procedure firmly binds small specimens to the filter (*cf.*, Hayat, 1978). Filters and tumor material were then dehydrated in graded ethanols and critical point dried from liquid CO_2. After examination with a compound microscope, sections were cut out, mounted on studs with double stick tape, sputter coated with Au/Pd, and photographed using ISI DS130 and Super IIIA scanning electron microscopes.

Infected and uninfected skeleton (1 to 3 mm diameter) previously dried at room temperature was thoroughly rehydrated in sea water and subjected to mechanical stress in a Scott tensile stress tester using step-wise loading. Elongation of the skeleton was recorded as a function of the original length. Cross sectional area of tumorous material was taken as the mean diameter above and below the infected region.

RESULTS

Algal morphology, ultrastructure, and pigmentation

The alga from intact tumors is typically found in masses composed of long, cylindrical filaments (Fig. 2A) composed of cells 4.5 to 6.5 μm in diameter. Cell junctions lack plasmodesmata. The cytoplasm is typically vacuolate, and while some of the vacuoles are filled with a loose fibrillar matrix (Fig. 2C), they are often free of

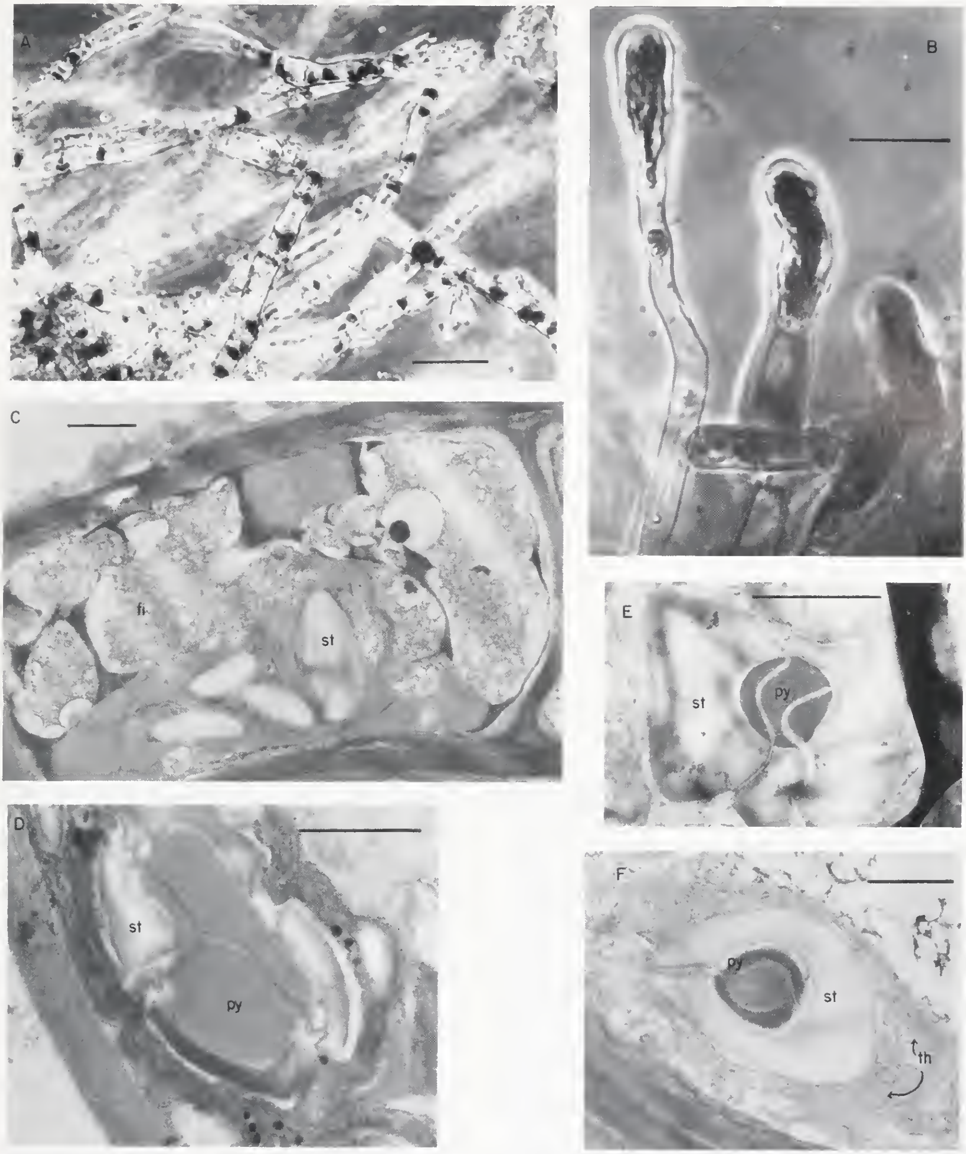

FIGURE 2. *Entocladia endozoica* field specimens. A. Vegetative filaments dissected from a nodule, scale bar is 30 μm. B. Filament ends from a nodule exposed to sea water for 10 days *in situ,* scale bar 30 μm. C. Transmission electron micrograph of a vegetative filament cell: fi = fibrilar matrix, st = starch, scale bar = 1.0 μm. D., E., and F. Pyrenoid structure: st = starch, py = pyrenoid, th = thylakoids, scale bars = 1.0 μm.

organic material. There are one or two chloroplasts which are always parietal and lack girdling lamellae. The thylakoid membranes are stacked into undulating lamellae of variable thickness. Part of the irregularity of the chloroplast is due to interruption of the lamellae by ellipsoidal starch grains (Fig. 2D, E, F) and by several pyrenoids. The pyrenoid matrix is surrounded by 2–4 starch plates and is either bisected or trisected by chloroplast membranes. In the latter case, the membranes either may be parallel, or perpendicular and confluent (Fig. 2D, E). While some additional irregularity may be due to the vagaries of fixation, such chloroplasts are typical of the Chlorophyta (Dodge, 1973; Pickett-Heaps, 1975).

Tumors broken open and exposed to ambient sea water contain algal filaments that terminate in larger, rounded cells (Fig. 2B). These terminal cells are distinguished by enlarged and more numerous chloroplasts which usually fill and eliminate the bulk of the intracellular spaces. Within the chloroplast, the number of starch grains is increased and oil droplets, mostly 0.1–0.15 μm diameter, become conspicuous and numerous (Fig. 3). As will be described below, these rounded cells are reproductive.

Acetone extracts of whole filaments contain chlorophylls a and b as determined by spectrophotometry (Fig. 4) and thin layer chromatography (Fig. 5). The extracts also possess several carotenoids including siphonaxanthin, neoxanthin, violaxanthin, lutein, and siphonein. These were identified by comparison with pigments extracted from the siphonous alga *Avrainvillea nigricans* Decaisne. The R_f values of the carotenoids from both algae were identical and corresponded to those given by Kleinig (1969) for *A. nigricans* and other siphonous algae.

Culture studies

Pure cultures of *Entocladia* were isolated using only nonreproductive material from intact tumors. Within two weeks on Provasoli's agar, the alga produced compact colonies composed of both prostrate and erect filaments (Fig. 6). When transferred to fresh liquid culture, these cells became ellipsoid and produced numerous chloroplasts (Fig. 7A). In nitrate and phosphate enriched media following medium replenishment,

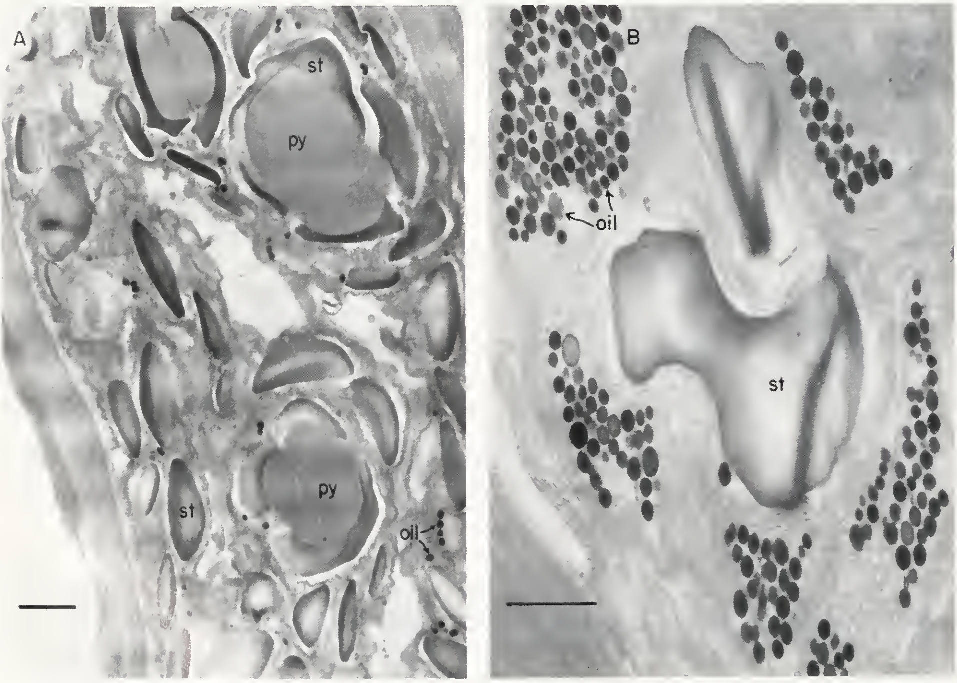

FIGURE 3. Numerous starch and oil droplets typical of reproductive cell chloroplasts, scale bars = 1.0 μm.

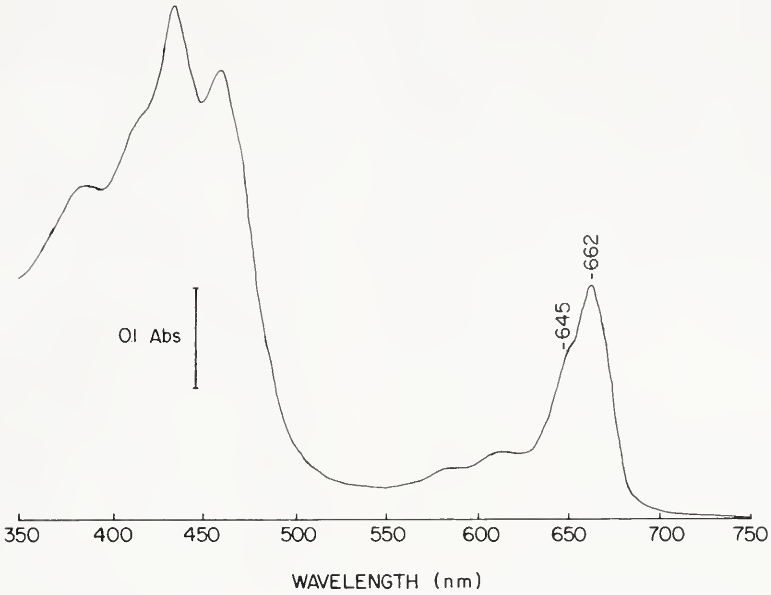

FIGURE 4. Spectrum of *E. endozoica* pigments (grown in culture) solubilized by 90% acetone.

these pre-reproductive cells enlarged and eventually became flask shaped (Fig. 7C, E) releasing swarmers. We have not determined whether these are zoospores or gametes. The swarmers were released through a single exit papilla (Fig. 7E) and possessed two flagella, but some had three and in one instance a pentaflagellated swarmer was observed (Fig. 8A, B, C).

Changes in inorganic constituents of the medium did not alter the ellipsoid morphology, although in phosphate and/or nitrate depleted media, setae were produced from bulbous extensions of the sub-polar regions (Fig. 7D). Organic carbon sources such as glucose, glycerol, acetate, glutamate, and aspartate were also ineffective. However, filaments grown in casamino acid-enriched media became elongated and produced filaments remarkably similar to those found in the tumors (Fig. 2A). The effect of casamino acids could be reproduced only by tyrosine or cystine at concentrations from 10^{-3} to 10^{-5} *M*.

Studies following the uptake and distribution of labeled substrates indicated that *Entocladia* took up carbon dioxide as well as amino acids from an amino acid mixture linearly over a one hour incubation period; glucose, glutamate and tyrosine were not taken up. After 1 hour, 89 to 96% of the HCO_3^- taken up was found in the cold TCA-insoluble fraction, and as expected, the amount of HCO_3^- taken up in the dark was only 1 to 4% of that taken up in the light (Table I). Amino acid uptake was small compared to HCO_3^-, and about 60% remained TCA soluble. Amino acid uptake was not affected by the presence of tyrosine in the growth medium and was only diminished 30–40% in the dark indicating that heterotrophy is not significant compared to the alga's autotrophic nutrition. These results have been duplicated with algal filaments isolated from freshly collected tumors (Makemson, unpub. data).

Effect on the host

The tumor-like swellings characteristic of the presence of *Entocladia* consisted primarily of the algal mass confined by laminated walls of host skeleton (Fig. 9B, C).

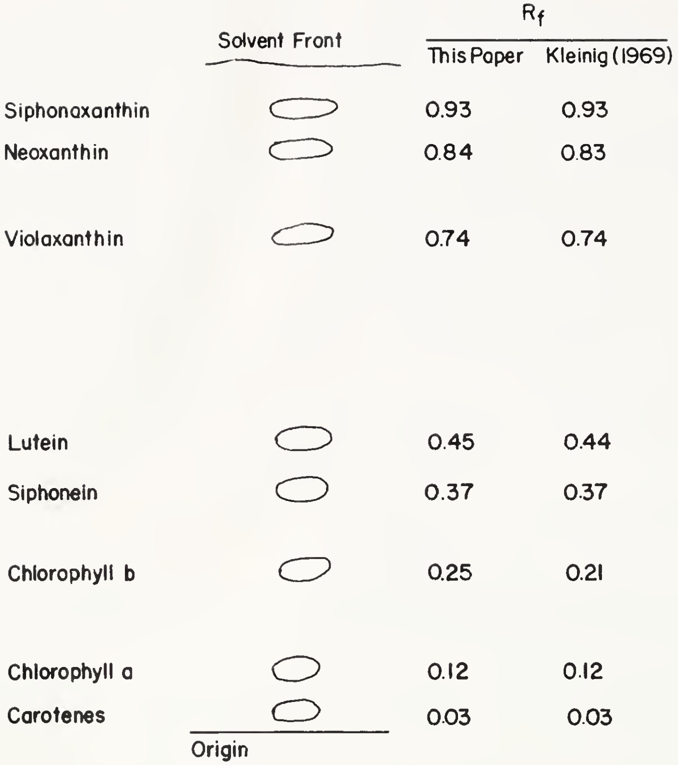
Solvent Front
Rf
This Paper
Kleinig (1969)
Siphonaxanthin 0.93 0.93
Neoxanthin 0.84 0.83
Violaxanthin 0.74 0.74
Lutein 0.45 0.44
Siphonein 0.37 0.37
Chlorophyll b 0.25 0.21
Chlorophyll a 0.12 0.12
Carotenes 0.03 0.03
Origin

FIGURE 5. Thin layer chromatography of acetone soluble pigments from *E. endozoica* on cellulose using methanol:acetone:water (30:10:3).

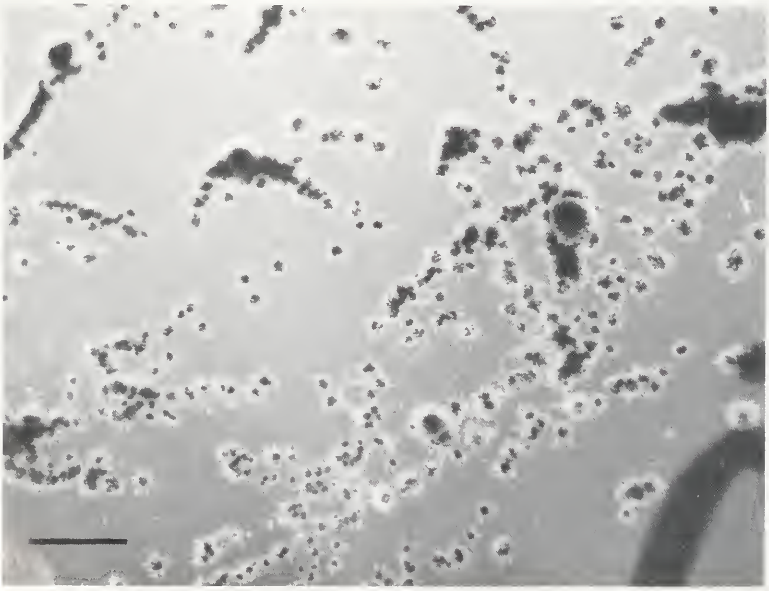

FIGURE 6. *E. endozoica* colonies on Provasoli's agar medium. Scale bar = 10 mm.

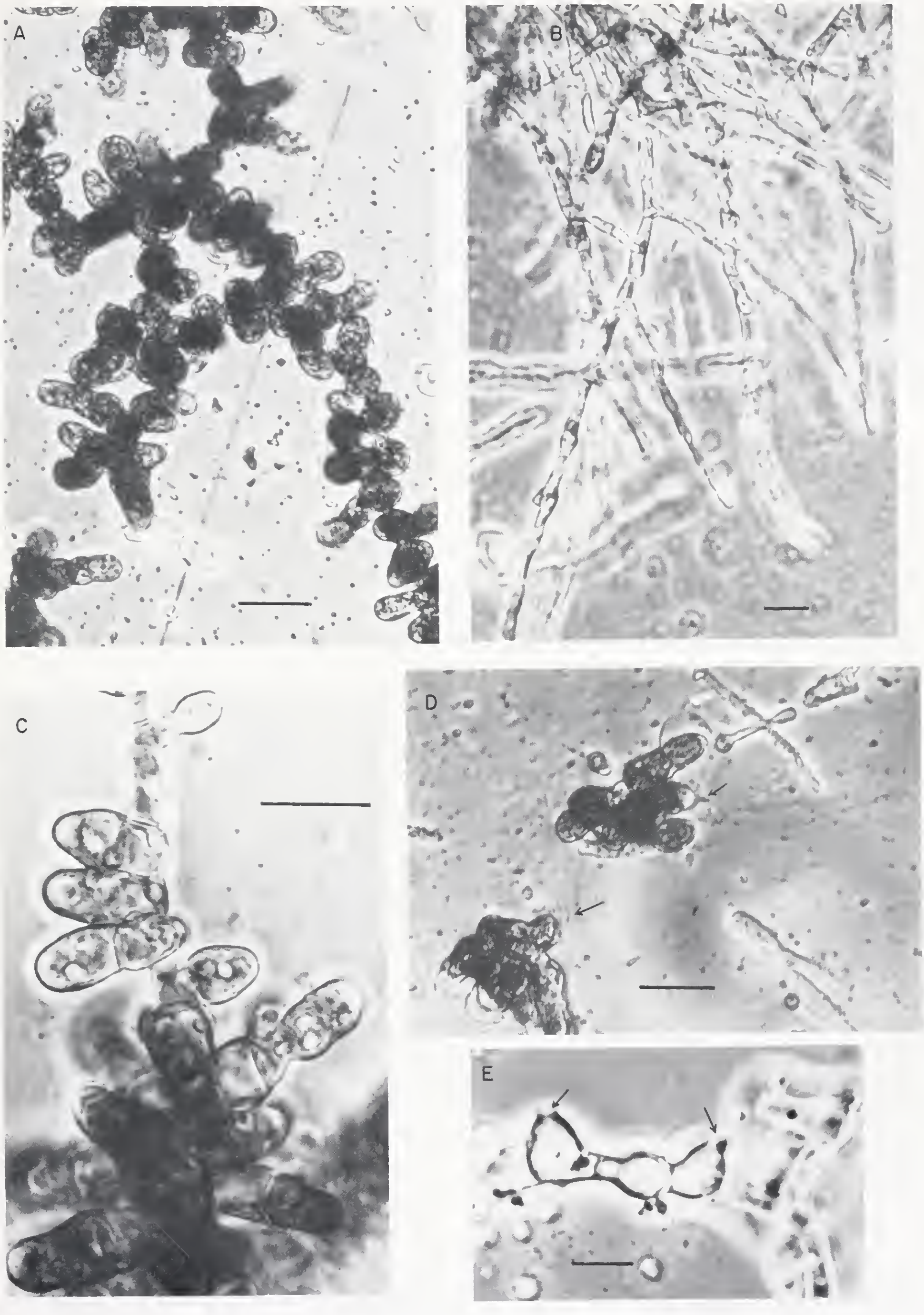

FIGURE 7. *E. endozoica* cells in pure culture. A. Pre-reproductive cells full of chloroplasts. B. Vegetative cells grown with tyrosine, 10^{-4} *M.* C. Intermediate stage in swarmer production. D. Setae originating from bulbous extensions of pre-reproductive cells (at arrows). E. Empty sporangia (or gametangia) with single exit papillae (at arrows). Scale bars = 30 μm.

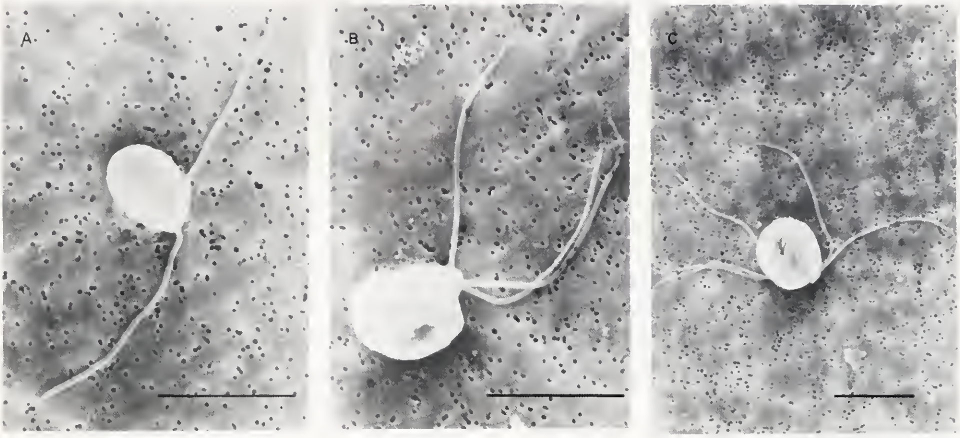

FIGURE 8. Scanning electron micrographs of swarmers possessing 2 (A), 3 (B), and 5 (C) flagella. Scale bars = 5 μm.

Much of the tumor histology is explainable simply by the mechanical strain of the algal mass and the increased surface area of host tissue that results from it. There was no pathology associated with the polyp layer surrounding the tumors. A study of reproduction in six normal and six infected colonies, for example, showed no differences in either the maturation or the development of gonadal material (Goldberg, unpub. data).

The major histopathology of the host involved production of abnormally large numbers of scleroblast cells and granular amoebocytes. The scleroblasts proliferated in the periaxial area (axial sheath) closest to the infected skeleton. Here the spicular weight per cm^3 of host tissue was 41.2% greater than in normal hosts ($n = 11$, $P = <0.03$). The resulting mass of purple sclerites appeared to form a loose calcitic capsule around the infected skeletal region (Fig. 9A). The size of the axial sheath sclerites was within the range found in uninfected specimens (110–214 μm), however, there appeared to be a greater proportion of the ornate, mature spicules in infected colonies.

The granular amoebocyte, normally present in small numbers, accumulated in the spaces of the periskeletal mesoglea when *Entocladia* was present (Fig. 10A). This occurred whether or not the alga was totally confined by host skeleton. When algal filaments extended beyond the skeletal boundary, the amoebocytes' numerous vesicles were released by a process that appeared to involve cell lysis (Fig. 10B), coating the algae with mesoglea like (fibrous, PAS-positive) material. The coated algae (Fig. 10C) were subsequently encapsulated by skeletogenic epithelium.

Skeletal mechanics

Comparison of stress/strain relationships in infected and uninfected skeletal material is shown in Figure 11. Normal *P. flagellosa* had a sigmoidal stress curve similar to keratin, antipathin, and other moderately cross-linked fibrous proteins (Wainwright *et al.*, 1976), but with apparently greater extensibility. The mean elastic modulus in the linear range was very close to 1. With continued stress the skeletal strain increased more rapidly, to an average ultimate tensile strength of 28 kg mm^{-2} (274 MN^{-2}). Uninfected skeletal material can extend to over double its original length in the

TABLE I

Uptake of $H^{14}CO_3^-$ and ^{14}C-amino acid mixture by cultures of Entocladia *filaments*

Culture[a]	Incubation[b]	DPM taken up[c]	f-moles/cell[d]
Provasoli's Broth +$H^{14}CO_3$	light	35838 total	47
		32218 TCA ppt	42
	dark	688 total	0.9
		102 TCA ppt	0.1
+^{14}C-aa's[e]	light	9486 total	0.11
		3860 TCA ppt	0.041
	dark	7075 total	0.11
		3159 TCA ppt	0.037
Provasoli's Broth + Tyrosine + $H^{14}CO_3$	light	32684 total	105
		31723 TCA ppt	102
	dark	513 total	1.6
		80 TCA ppt	0.26
+^{14}C-aa's	light	9151 total	0.26
		2721 TCA ppt	0.08
	dark	6096 total	0.17
		1646 TCA ppt	0.047

[a] Cultures were vegetative cells grown in Provasoli's medium with and without 1 m*M* tyrosine. Cells were harvested and resuspended in broth without tyrosine; culture grown without tyrosine contained 9 × 10^4 cells/ml and the tyrosine-grown culture contained 4 × 10^4 cells/ml.

[b] Incubation of 5.0 ml of cell suspension in flasks with the appropriate label added as 20 μl. Dark incubation was in foil-wrapped flasks; both incubated at 27°C at 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ using cool white fluorescent illumination for 1 hour.

[c] Radioactivity taken up was determined by filtration of 2 ml of incubation mixture onto 0.45 μm pore size filters, rinsed with three washes of artificial sea water (total) or 5% TCA (TCA ppt), and placed immediately into 5 ml of Hydromix scintillation mixture. Counts per minute were corrected for counting efficiency, background, and quench.

[d] Specific activity of each label was used to calculate amounts of material taken up and divided by the average number of cells on each filter.

[e] L-amino acid mixture, NEC-445 New England Nuclear, Boston, Massachusetts, contains 15 amino acids in the same relative proportions as found in a typical algal protein hydrolysate. 0.1 mCi/ml used undiluted with cold amino acids.

hydrated state. Skeletal material infected with algae, on the other hand, showed a mean decrease in the tensile strength of over 60% and a decrease in elasticity of over 90%.

DISCUSSION

Systematic assessment

Entocladia's thallus morphology, pigment composition, and chloroplast ultrastructure are typical of the Chlorophyta. However, its assignation to lower taxa such as the Chaetophoraceae is a matter of opinion (Bold and Wynne, 1978). Certain marine chaetophoracean genera have been placed recently in the restructured family Ulvellacea based upon their life history, chloroplast pigments and ultrastructural features (O'Kelly and Yarish, 1981; O'Kelly, 1983; O'Kelly and Floyd, 1983). The

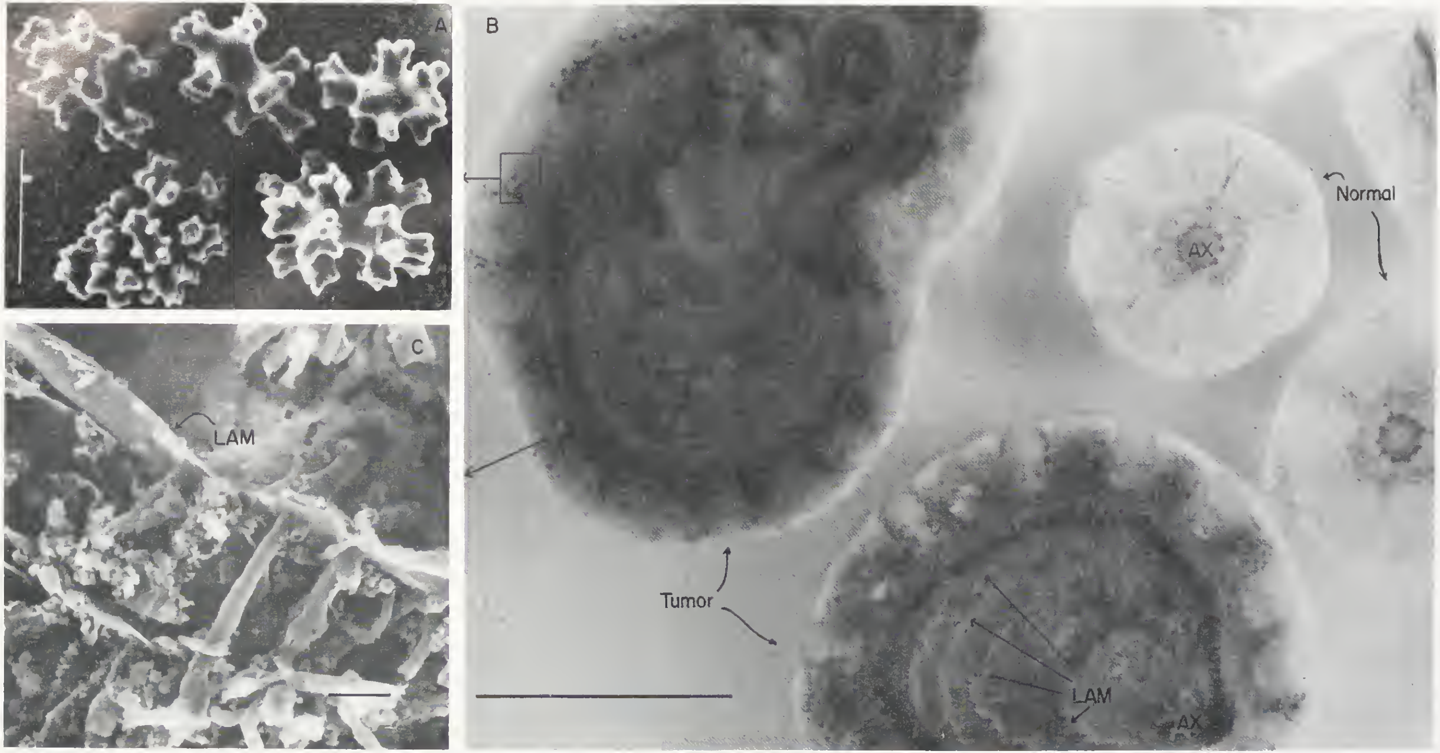

FIGURE 9. Comparison of normal and infected corals. A. Spicules are 40% more abundant in the infected condition on a weight per unit tissue volume basis. Scale bar = 100 μm. B. Cross sections comparing infected material with normal *P. flagellosa* (upper right). Laminations (lam) of axial skeleton are separated by radially oriented algal filaments. Uninfected skeleton (ax) is solid; individual laminae are fused. Scale bar = 50 μm. C. Scanning electron micrograph of algae and interposed host skeleton. Scale bar = 50 μm.

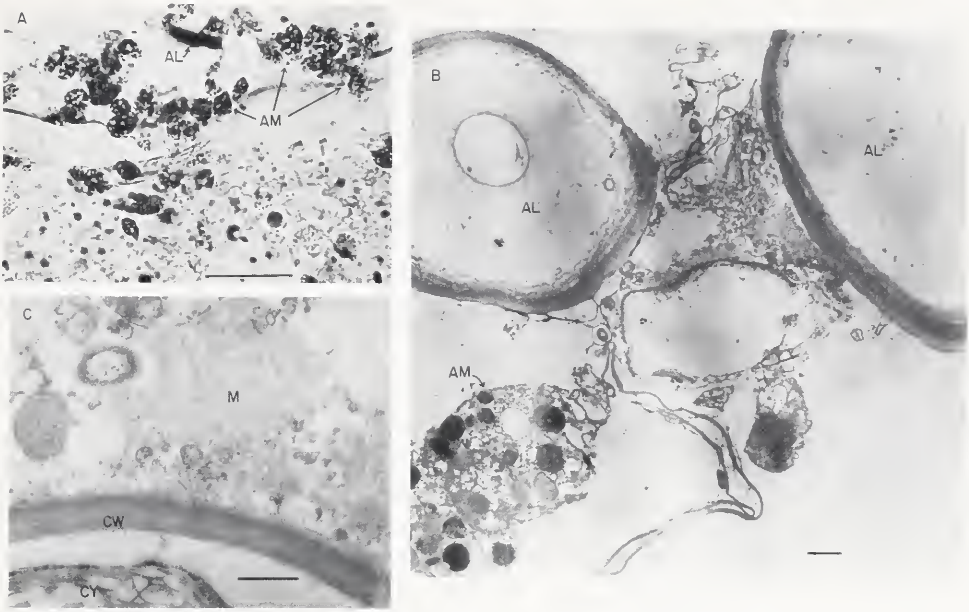

FIGURE 10. Association of coral amoebocyte and algal filaments. A. Light micrograph of amoebocytes aggregated near an algal filament; note PAS-positive vesicles; scale bar = 50 μm. B. Low power electron micrograph of amoebocyte lysing and releasing contents near two algal filaments; scale bar = 1 μm. c. Mesoglea coating an algal filament; scale bar = 1 μm. AL, algae; AM, amoebocyte; CW, cell wall; CY, chloroplast; M, mesoglea.

latter authors give familial characters that agree with our material. These include branching, filamentous, chlorophytic microalgae that may form pseudoparenchymatous masses with uninucleate cells containing parietal chloroplasts with one or more pyrenoids; colorless, nonseptate, anucleate setae may be present depending upon environmental conditions, especially nutrient depletion. Ultrastructural characteristics first described by Stewart *et al.* (1973) indicate that ulvellacean genera may also be distinguished by their lack of plasmodesmata and the presence of traversing thylakoid membranes in the pyrenoid. Our material agrees with this diagnosis (Fig. 2D, E, F) but is clearly different (segregative cell division) from the siphonaceous alga found in *Gorgonia* by Morse *et al.* (1977, 1981).

The genus *Entocladia* has been circumscribed by O'Kelly and Yarish (1981). Our material conforms to their characteristics with the exception that the number of flagella appears more variable. Occurrence of tri- and pentaflagellate forms may be an artifact of culture and preparation. *E. endozoica* probably produces bi- and quadriflagellate motile cells which are characteristic of this group (Yarish, 1975; Nielsen, 1979; O'Kelly and Yarish, 1980, 1981). The pigments of the genus *Entocladia* include chlorophylls a and b, carotenes, lutein, neoxanthin, siphonaxanthin, and violaxanthin (O'Kelly, 1982). The characters that distinguish *E. endozoica* from other *Entocladia* species are (1) amino acid-induced morphological changes; (2) its endozoic habitat; and (3) presence of siphonein in addition to the other carotenoid pigments.

Entocladia endozoica Goldberg, Makemson and Colley sp. nov.

> *Fila cylindrica 5–10 μm diametro, 9–18 μm longa unum chloroplastum parietalem lobatum habantia, uno vel aliquot pyrenoidibud bi- vel tri-lenticularibus ubi intra hospitem Pseudoplexauram. Fila aquam marinam exposita vel in substrata*

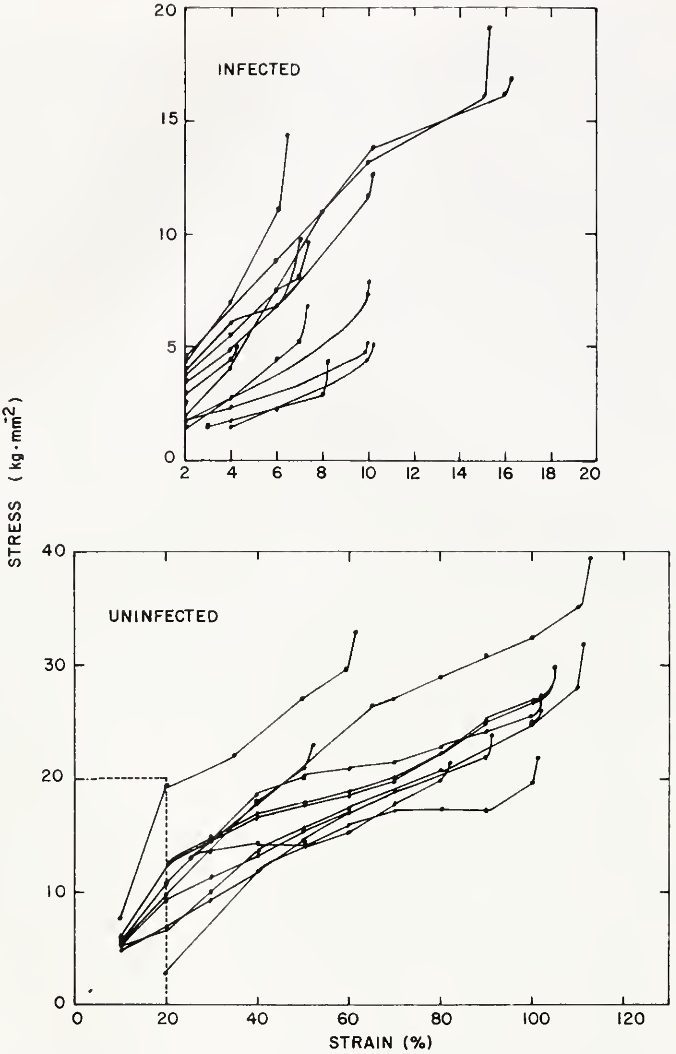

FIGURE 11. Stress-strain curves of uninfected and infected skeletons. The curves for the infected skeletons (A) fit within the dashed area of the uninfected material (B).

nutricia autotrophica sativa cellulas globosas 11.5–18 μm diametro chloroplastis multos grana multa amyli et guttulas olei signati. Sub statis phosphatis vel nitratis effoetis hae cellulae setas quoque procreare possunt. Cellulae globosae cellulas ampulliformes procreantes greges ellipsoideos 2.3–3.2 μm latos, 3.4–4.0 μm longos, 2-, 3- vel raro 5-flagellis fiunt. Cellulae reproductivae papilla singula exitus. Pigmenta chlorophylla a et b, siphonaxanthin, violaxanthin, siphonein, neoxanthin, et lutein includent. Habitat endozoica fortasse specifica generi Pseudoplexaurae.

Entocladia endozoica sp. nov.: cylindrical filaments 5–10 μm in diameter and 9–18 μm in length possessing one parietal, lobed chloroplast with one or several bi- or trilenticular pyrenoids when within its coral host. Filaments exposed to sea water or cultured in autotrophic media produce rounded cells 11.5–18 μm in diameter with multiple chloroplasts characterized by numerous starch grains and oil droplets. Under conditions of phosphate or nitrate depletion these cells may also produce setae. Rounded cells become flask-shaped producing ellipsoid swarmers, 2.3–3.2 μm wide and 3.4–4.0 μm long with 2, 3, or rarely 5 flagella. A single exit papilla characterizes these reproductive cells. Pigments include chlorophylls a and b, siphonaxanthin, violaxanthin, siphonein, neoxanthin, and leutein. Habitat endozoic, possibly specific to gorgonian corals of the genus *Pseudoplexaura*.

Type Locality: Bache Shoals, northern Florida Keys, depth 3 m. Holotype specimens have been deposited at the National Herbarium (Smithsonian Institution, Washington, DC 20560) and pure cultures with the Culture Collection of Algae of the University of Texas (Austin, TX 78712).

Biological significance

The peculiar habitat of *E. endozoica* has in addition to systematic importance, relevance to the coelenterate host response as well as regulation of the algal life cycle. Histological evidence shows that the bulk of the abnormal swellings consist of living algal filaments enclosed by gorgonian skeletal protein. Some specimens show filaments extending into the cellular (mesogleal) areas of the host whereupon they become surrounded by granular amoebocytes. Although the precise function of these host cells is unclear, this interaction seems to be associated with encapsulation. Thus, what appear to be algal filaments penetrating the skeleton (Fig. 9C) are more likely algae in the process of being covered or surrounded by skeletal material. The fact that the algae are only occasionally able to break free from this matrix may be indicative of an efficient host response rather than an algal-induced disruption of host skeletogenesis. The proliferation of spicular material may serve as reinforcement for the capsule, preventing the algae from interacting with the polyp layer. Nonetheless, from the viewpoint of host defense mechanisms, the response is only partly competent since the alga remains viable within the host skeleton.

In the enclosed tumor, *E. endozoica* filaments are uniformly elongated. These vegetative filaments grow only in media enriched by casamino acids, cystine or tyrosine in particular, the latter without being accumulated by the cells. Since gorgonian skeletons concentrate tyrosine (Goldberg, 1976, 1978) it is possible that the skeletal milieu is tyrosine-enriched, thereby exerting a regulatory rather than a nutritional role in algal morphology and reproduction. This is supported by the miniscule uptake and incorporation of radiolabeled amino acid mixtures (Table I) and tyrosine.

Growth and proliferation of *Entocladia* filaments result in host skeletal dysplasia, with consequent reduction in both the tensile strength of the skeleton and its extensibility. Although we have not performed other measurements such as cyclic stress-strain and stress relaxation tests, the data suggest that turbulent flows or other forms of mechanical stress are likely to cause preferential breakage of the coral colony at the tumor site. Open tumors can be found in normal field conditions and always contain rounded reproductive filaments (Fig. 2). Tumors cut open artificially, exposing the vegetative filaments to sea water, produce reproductive filaments within ten days; under laboratory conditions only four days are required. Sea water as a cystine-tyrosine depleted medium appears to be an essential factor in this conversion both *in situ* and in the laboratory. The other factor is a considerable increase in light intensity. A tumor cross section 1 cm thick (with external host tissue) reduces ambient light intensity by over 99%. Assuming 120,000 lux as an average condition, (cloudless day, zenithal sun) for surface irradiance (Sverdrup *et al.,* 1942) and a 40% reduction by 3 meters of type III ocean water (Gordon and Dera, 1969), the average amount of light penetrating a tumor should be less than 720 lux. Exposure by tumor breakage should suddenly expose algal filaments to 2 orders of magnitude more light. Experiments involving order of magnitude increases in light greatly increase growth rates (data not shown) but fail to produce reproductive cells, reinforcing our suggestion that nutritional factors, especially the lack of tyrosine or cystine, play a key role in initiating the reproductive process.

Although not observed in nature, motile cells are likely released from opened tumors before host tissue regenerates, closing over what has become an apical tumor. During the summer months, open tumors 2–3 cm in diameter heal completely within 7–10 days. Thus regeneration as well host reproduction (described above) appear to be unaffected by this association. Moreover, there is no evidence that *Pseudoplexaura* suffers from peripheral necrosis or erosion such as that described from algal-infected sea fans by Morse *et al.* (1977). Our previous study of the ecology and distribution of the tumor phenomenon (Goldberg and Makemson, 1981) showed no obvious correlation with known environmental stress areas in Florida. Furthermore, we have no evidence to indicate that the relationship described here is in the long run harmful to the host. In fact, broken *Pseudoplexaura* branches may result in vegetative propagation of the coral (*cf.,* Walker and Bull, 1983), although we have no data on branch survival. These observations suggest a close relationship between pathogen and host: *E. endozoica* is poised to respond quickly to autotrophic conditions and relatively high light fields; this exposure in turn, is made possible by the vegetative growth of the alga and the resulting loss in host skeletal strength. Further work will be necessary to determine if various pathogenic algae are genus-specific as first suggested by Morse *et al.* (1981). Such studies should provide fresh insights into the newly emerging field of coral reef pathology and open new avenues of research in alga-invertebrate symbioses.

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PRECOCIOUS MATURITY OF THE MAJID CRAB, *PUGETTIA PRODUCTA*, PARASITIZED BY THE RHIZOCEPHALAN BARNACLE, *HETEROSACCUS CALIFORNICUS*

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ABSTRACT

Mean size of adult kelp crabs, *Pugettia producta*, parasitized by the rhizocephalan barnacle, *Heterosaccus californicus*, was significantly less than the mean size of unparasitized crabs. This size difference resulted from parasitized animals having passed through fewer instars before the molt of puberty. Laboratory and field evidence indicated that parasitized crabs matured earlier than unparasitized ones. Precocious maturity of parasitized crabs fitted a model which postulated that size at maturity was determined by the value of a size-threshold. Crabs would not undergo the molt of puberty unless larger than the size-threshold. The value of the size-threshold would be influenced by environmental factors which could vary seasonally within one locality. Precocious maturity can be explained as a greater sensitivity of parasitized crabs to the stimuli that induce maturation above the size-threshold. The implication of this study is that size differences among populations of adult crustaceans may be accounted for by variation in the number of juvenile instars as well as size-selective predation and differential growth.

INTRODUCTION

The Rhizocephala are a distinctive order of barnacles which parasitize decapod crustaceans. In addition to inhibiting host reproduction and feminizing male hosts (see Hartnoll, 1967, for review), the parasite may affect adult host size. For example, Reinhard (1950, 1956) observed that *Callinectes sapidus* parasitized by mature *Loxothylacus texanus* were smaller than unparasitized adults and considered this to be due to hyperfeminization, a condition in which parasitized hosts precociously developed the broad adult female abdomen. Hartnoll (1967) termed this effect precocious maturity. Allen (1966) observed a similar phenomenon with another crustacean parasitic castrator, reporting that pandalid shrimp parasitized by a bopyrid isopod precociously developed breeding dress. A smaller mean size of parasitized hosts which may indicate precocious maturity has been noted for many sacculinid-host associations: Smith (1906), Okada and Miyashita (1935), Veillet (1945), Reinhard (1950), Overstreet (1978), and O'Brien (this study). In contrast, Philipps and Cannon (1978) reported no effect of a sacculinid on host size while Day (1935) and Vernet-Cornubert (1958) documented interactions in which sacculinid prevalence increased with increasing host size.

Interactions other than precocious maturity occur in other rhizocephalan families (see O'Brien and Van Wyk, 1984, for review). Brinkmann (1936) observed slower growth of a cohort of galatheids parasitized by a lernaeodiscid rhizocephalan than the unparasitized cohort. In the rhizocephalan family Peltogastridae (Bourdon, 1963)

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and perhaps the Lernaeodiscidae (Veillet, 1945), a positive correlation between host size and parasite prevalence has been observed: a pattern that is frequently associated with parasitic castrators (Kuris, 1974; Baudoin, 1975; Kuris *et al.,* 1980).

The existence of other interactions between rhizocephalans and their hosts, while not necessarily precluding precocious maturity, may mask it and make precocious maturity difficult to detect. Alternatively, it can be questioned whether precocious maturity actually is the appearance of adult structures early in development, as the term 'precocious' implies, if the only evidence is a smaller size of parasitized adult hosts. Many causes of size differences among groups of crustaceans have been reported: differential growth (Kinne, 1959; Newman and Pollock, 1974), size selective predation (Brooks and Dobson, 1965), and season of maturation (Kuris, 1971) to name just a few. Yet, other than documenting size differences between parasitized and unparasitized crabs, no study has provided evidence that infection by a sacculind actually resulted in the precocious acquisition of the adult morphology by the host.

This study demonstrated causal factors leading to smaller adult mean size of the majid crab, *Pugettia producta,* parasitized by the sacculinid, *Heterosaccus californicus.* Infection did not alter molt increments from those of unparasitized animals, but did reduce the number of molts before the molt of puberty. This is the first study to document with laboratory and field evidence precocious maturity in hosts parasitized by Rhizocephala.

MATERIALS AND METHODS

Pugettia producta individuals were collected monthly from Coal Oil Point, Goleta, Santa Barbara County, California from November 1976 to September 1982. Crab carapace length was measured to the nearest 0.1 mm from the tip of the rostrum to the midline of the posterior carapace margin. Sexual maturity of the crabs was determined from allometric changes of the male chela and from female abdominal width associated with the molt of puberty (Pérez, 1928; Hartnoll, 1967; O'Brien, in prep). Crabs were individually marked using the position of holes punched in the exoskeleton (Born, 1970; Kuris, 1971). Adult crabs were recorded as parasitized if they possessed either the external stage (externa) of the rhizocephalan or a necrotic scar on the abdomen where the externa had detached from the host (Lützen, 1981). All crabs with a compressible merus and parasitized crabs with externa lacking a mantle opening were assumed to have matured in the month of collection. The population structure of adult crabs was determined from records of first capture only.

Laboratory growth studies were conducted at the Marine Laboratory of the University of California, Santa Barbara. Crabs were maintained individually in 0.5 to 2.25 liter perforated polyethylene freezer containers depending on size of crab. The containers were submerged in ambient flowing sea water. Crabs were inspected daily and fed the kelp, *Egregia laevigata.* Only first laboratory molts of crabs of comparable size missing no more than one leg were used in the statistical analyses. In an experiment designed to determine if there was a difference in the number of molts to maturity between parasitized and unparasitized crabs, a cohort of crabs was maintained through two laboratory molts. To minimize variability, all crabs in this experiment were collected at the same time (one tidal series in May 1979) at the same place (Coal Oil Point) from the same alga (*E. laevigata*) and were of similar size (29.5–34.7 mm). Consecutive instars of these crabs were counted until the parasitized crabs matured. Statistical analyses were conducted according to Sokal and Rohlf (1981), except for separation of modes by probability graph paper (Cassie, 1954). Unbalanced two-way ANOVA and Duncan multiple range tests were computed by the UCSB ITEL AS/6 computer using Statistical Analysis System (SAS) (Helwig and Council, 1979).

Comparisons of linear regressions were done by ANCOVA based on a program from Zar (1974).

RESULTS

Host size distribution

No parasitized host was found larger than 85 mm. Only 0.4% of the parasitized population (3 crabs) was larger than 80 mm, although 11.7% of the unparasitized adult population exceeded this size (Fig. 1). Mean size of parasitized adults [59.2 mm ± 7.8 (S.D.) (n = 730)] was significantly less than the respective mean sizes of unparasitized adult females and males [66.5 mm ± 9.4 (n = 1846) and 67.8 ± 12.6 (n = 1615)] ($P < 0.001$, Student's t-test).

Using probability graph paper the size frequency distribution of parasitized adult crabs was separated into three modes. The first mode had a mean of 56 mm and comprised 62% of the parasitized population. Succeeding modes had means of 67 and 79 mm constituting 35% and 3% of the population respectively. Percentage of hosts parasitized (prevalence) within the total adult population was 17.4%. Prevalence decreased with increasing host size above a carapace length of 57 mm (Table I). Almost 90% of the parasitized population was found between 46 and 69 mm carapace length (Table I).

Molt increment

Linear regressions of molt increments (post-molt length − pre-molt length) of immature crabs as a function of pre-molt carapace lengths were: parasitized crabs ($Y = 0.283x + 1.255$, $r = 0.876$, $n = 22$), unparasitized females ($Y = 0.356x - 0.854$, $r = 0.939$, $n = 75$) and unparasitized males ($Y = 0.293x + 0.333$, $r = 0.922$, $n = 65$). When subjected to ANCOVA at $P = 0.05$, no significant difference between these regressions in slope or elevation was found whether parasitized crabs were

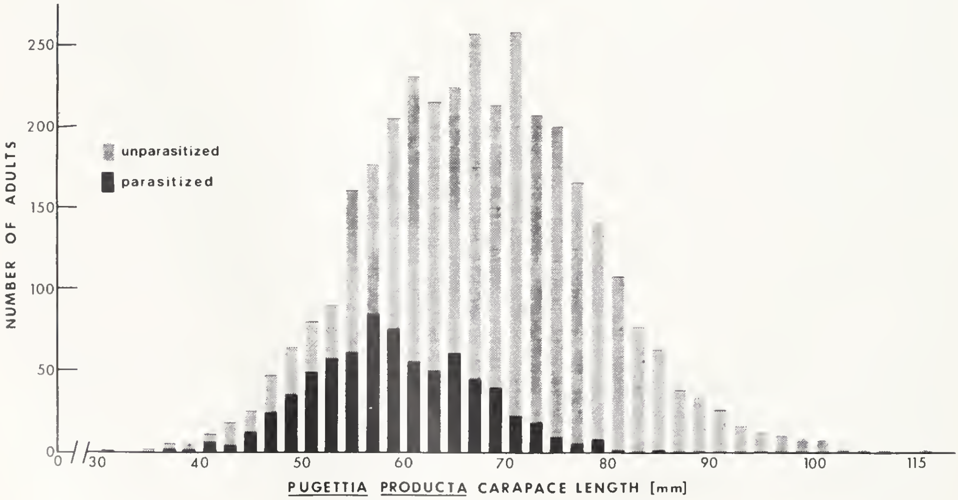

FIGURE 1. Size-frequency distribution of unparasitized *P. producta* adults and adults parasitized by *H. californicus.* Data were collected from Coal Oil Point from 1975–1982. Values for parasitized animals are superimposed upon those of unparasitized animals.

TABLE I

Prevalence (%) and size-frequency distribution of adult H. californicus *within size-classes of the adult* P. producta *population at Coal Oil Point California*

Host carapace length (mm)	<45	46–57	58–69	70–81	82–93	>94
Percent of *P. producta* parasitized (n = 4,186)	27.2	31.7	19.6	5.5	0.8	0.0
Percent of *H. californicus* population (n = 729)	3.4	42.7	45.0	8.6	0.3	0.0

compared with unparasitized females (Fig. 2) (slope F = 2.88, y-intercept F = 0.421) or unparasitized males (slope F = 0.375, y-intercept F = −3.151).

The linear regression which was computed for molt increments at the molt of puberty against carapace length for parasitized crabs was significant (Fig. 3) (r = 0.603, critical value at $P = 0.01$ was r = 0.575). However, if the data point for the largest parasitized crab (54.3, 18.0) was omitted from the computation, the correlation of the resulting linear regression (r = 0.392) was not significantly different from r = 0. The comparable regressions for unparasitized females (r = 0.37) and unparasitized males (r = 0.021) were also not significantly correlated. Molt increment at the molt of puberty was not correlated with pre-molt size. Comparing all observed increments

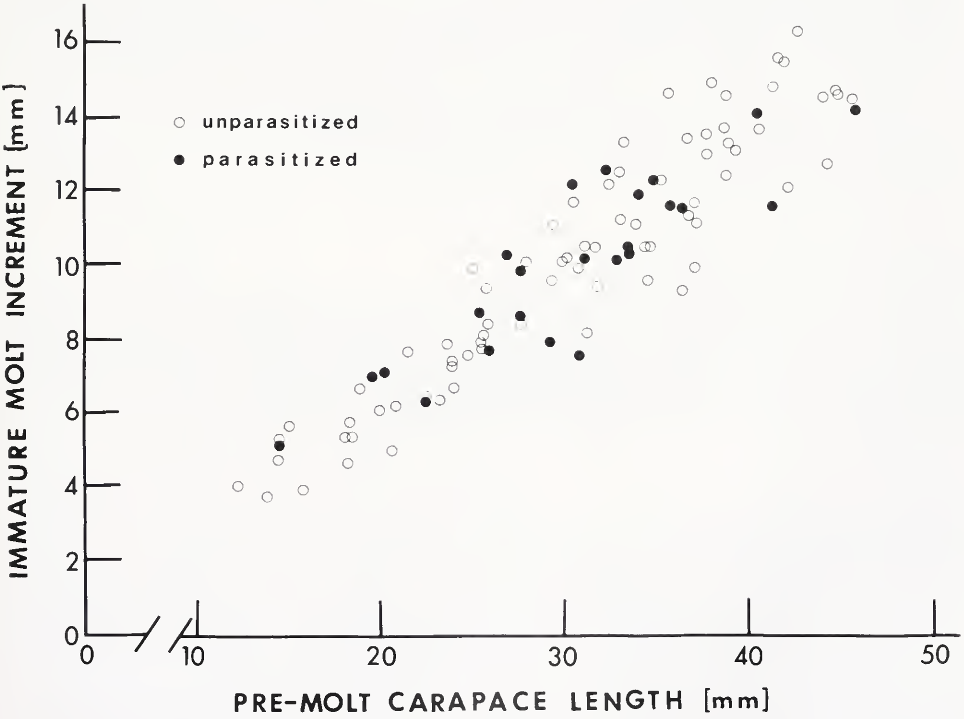

FIGURE 2. Increase in size at the first laboratory molt for parasitized juvenile female *P. producta* and of juveniles parasitized by *H. californicus* as a function of pre-molt carapace length.

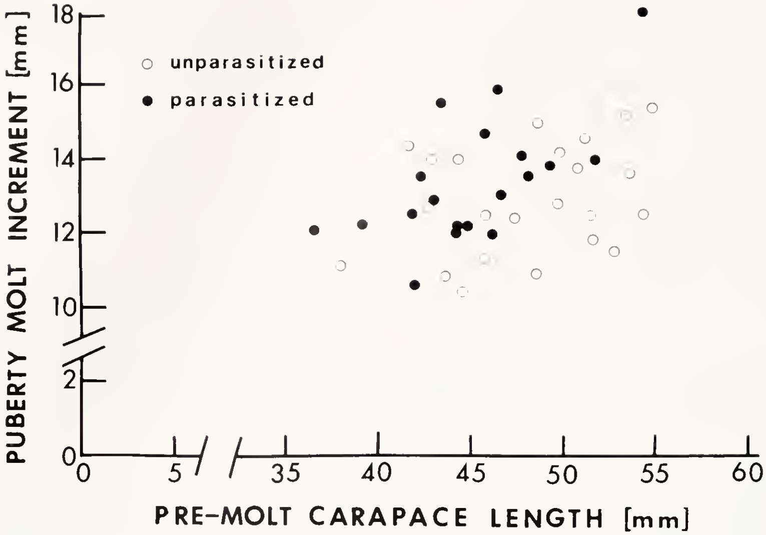

FIGURE 3. Increase in size at the molt of puberty for unparasitized female *P. producta* and of individuals parasitized by *H. californicus* as a function of pre-molt carapace length.

at the molt of puberty using the Mann-Whitney U-test revealed no significant difference between parasitized and unparasitized females ($P > 0.20$) nor between parasitized and unparasitized male crabs ($P > 0.10$). Linear regressions of log-log transformations of pubertal molt increments were also determined for unparasitized females ($y = 0.4693x + 0.321$; $r = 0.365$) and parasitized crabs ($y = 0.7903 - 0.1844$; $r = 0.578$). ANCOVA revealed no significant difference between slopes ($0.50 > P > 0.25$, $F = 0.698$) nor intercepts ($0.10 > P > 0.05$, $F = 3.301$) of the linear regressions of the transformed data.

Number of molts to maturity

The first laboratory molt of the entire cohort of parasitized and unparasitized crabs was an immature molt. Of those individuals molting a second time, a significantly greater proportion of parasitized crabs underwent the molt of puberty than of unparasitized crabs. ($P = 0.005$, G-test of independence) (Table II).

TABLE II

Maturation state of similar-sized P. producta *following a second laboratory molt*

	Number of adults	Mean length (mm) Pre-molt	Mean length (mm) Post-molt	Number of juveniles	Mean length (mm) Pre-molt	Mean length (mm) Post-molt
Parasitized	5	43.8 ± 3.38*	53.3 ± 2.42	1	43.7	56.0
Unparasitized	1	47.7	56.3	10	42.1 ± 1.79	50.5 ± 3.24

* = Student's *t*-interval, $P = 0.05$.

Instar duration

Instar durations of unparasitized male and female crabs undergoing immature molts were not significantly different ($P > 0.40$, Mann-Whitney U-test) nor did they differ significantly from instar durations of parasitized crabs (Fig. 4) ($P > 0.30$, Mann-Whitney U-test). Similar comparisons of instar durations preceding molts of puberty revealed no significant differences between unparasitized males and females nor between parasitized and unparasitized crabs ($P > 0.10$).

Maturation season

About 90% of the molts of puberty observed in the field and laboratory occurred during the period from May through October, the maturation season (Fig. 5). Throughout the maturation season when a parasitized crab larger than 30 mm molted, it was much more likely to undergo the molt of puberty than a concurrently molting unparasitized crab. Thus, the relative frequency of pubertal molts (total number of pubertal molts divided by the number of all observed molts × 100) for crabs larger than 30 mm was significantly greater for parasitized than unparasitized crabs ($P < 0.001$, G-test of independence) (Fig. 6). In addition, size at maturity was strongly correlated with both the presence of a parasite (F = 23.54, $P < 0.0001$) and month in which maturation occurred (F = 3.93 $P < 0.01$, ANOVA) (Table III). Furthermore, as the maturation season progressed there was a significant difference in how mean size at maturity changed over time, decreasing for parasitized while increasing for unparasitized crabs ($P < 0.006$, F = 4.39, ANOVA). Analysis by the *a posteriori* Duncan's multiple range test at $P = 0.05$ revealed no significant differences in size at maturation for parasitized and unparasitized crabs maturing in May and June, but during the periods July–August and September–October mean size of newly

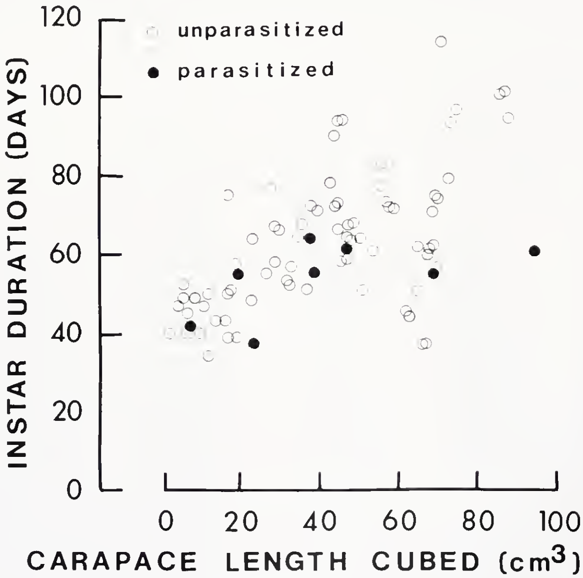

FIGURE 4. Instar duration of unparasitized juvenile *P. producta* and of juveniles parasitized by *H. californicus* plotted as a function of the cube of the pre-molt carapace length.

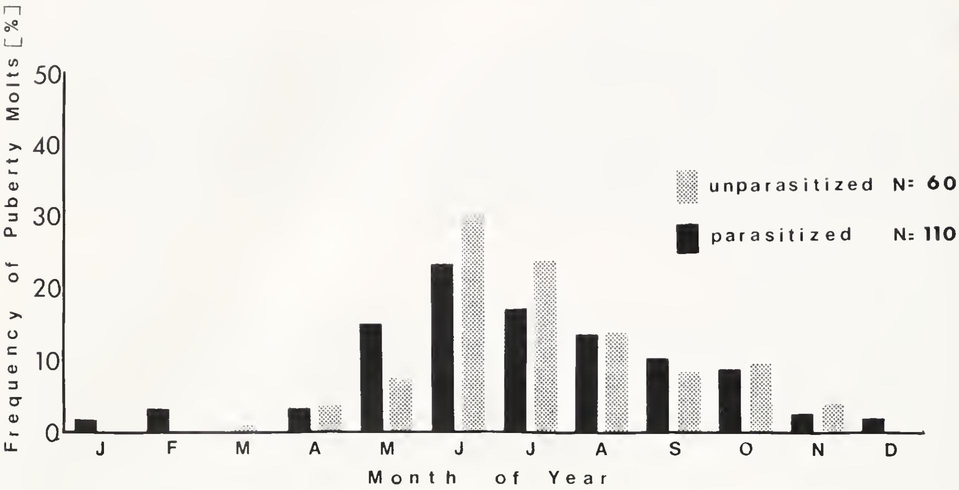

FIGURE 5. Relative frequency of molts of puberty from 1975–1982 computed as a percentage of the total number of puberty molts observed (N). Monthly values were computed independently for unparasitized *P. producta* and for crabs parasitized by *H. californicus*. Data were from monthly samples from Coal Oil Point.

matured parasitized crabs was significantly less than that of unparasitized crabs (Table III).

DISCUSSION

Prevalence of the rhizocephalan parasite, *Heterosaccus californicus*, was negatively correlated with increasing host size (Table I). No large adult crabs (>85 mm) were

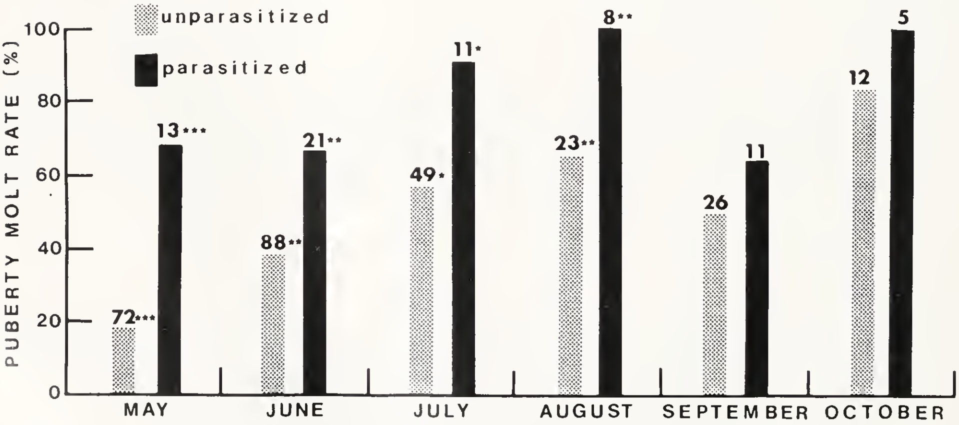

FIGURE 6. Relative rate of molts of puberty (total number of molts of puberty divided by total of all molts times 100) for crabs greater than 30 mm pre-molt carapace length. Numbers above each column indicate total number of molts observed for that crab group in that month. Data were accumulated from 1975 to 1982. Asterisks indicate significant differences between within month values at the following levels: $^{***}P < 0.001$, $^{**}P < 0.025$, $^{*}P < 0.05$, and lack of an asterisk indicates $P > 0.05$ (G-test of independence).

TABLE III

Size at maturity of parasitized and unparasitized P. producta *by months in which maturation occurred*

	Month	May–June	July–August	September–October
	n	44	42	19
Unparasitized	Size-range	50.1–77.2	54.3–86.2	54.8–94.9
	Mean size (mm CL)	62.8 ± 2.12*	65.0 ± 2.89	67.2 ± 4.29
	n	23	18	11
Parasitized	Size-range	51.4–78.7	38.8–63.0	50.5–62.0
	Mean size (mm CL)	60.7 ± 2.68	52.4 ± 3.08	57.9 ± 2.83

* = Student's *t*-interval, *P* = 0.05.

parasitized. However, the lower size limits of both parasitized and unparasitized adults were similar (Fig. 1). This study considered five possible explanations: 1) higher mortality of large parasitized crabs, 2) differential growth of parasitized and unparasitized adults, 3) differential growth of parasitized and unparasitized juveniles, 4) an environmental factor independent of the parasite, and 5) precocious maturity of parasitized hosts for the observed size differences between parasitized and unparasitized crabs.

A mortality rate of large parasitized crabs greater than that of unparasitized crabs seemed an unreasonable explanation for the observed size differences because no large (>85 mm) parasitized crabs were ever found. Sampling was conducted monthly for seven years and if significant numbers of large parasitized crabs had matured, some probably would have been detected, even if they had died shortly after maturation. In addition, parasitized crabs matured in the laboratory over the same size range as those found in the field; the largest newly matured parasitized crab (78 mm) was almost as large as the largest parasitized crab collected (85 mm). Both laboratory and field evidence corroborated on a maximum size of adult parasitized crabs which was about 20% (20–25 mm) less than the maximum size of unparasitized crabs.

Differential growth of adult crabs was rejected as an explanation for the observed size differences because most majid crabs including, *P. producta,* do not molt after the molt of puberty (Tessier, 1935; Carlisle, 1957; Hartnoll, 1963; Born, 1970). The smaller size of parasitized crabs must have resulted from events that occurred during the juvenile growth phase.

Two workers have examined the effect of sacculinids upon growth of juvenile crabs. Veillet (1945) concluded that the smaller size of *C. maenas* parasitized by *Sacculina carcini* was caused in part by reduction of the molt increment of large juvenile crabs. Veillet observed no difference between molt increments of small parasitized and unparasitized juveniles but did note a difference in larger sizes. Since the energy demands of adult rhizocephalans might sufficiently drain the host's lipid reserves and negatively affect host growth (Smith, 1906), Veillet theorized that immediately following infection *S. carcini* was too small to influence host growth. Veillet suggested that as the parasite grew it exerted an increasingly negative effect upon host growth; thus, only large parasitized juveniles displayed relatively small molt increments. In contrast Vernet-Cornubert (1958) concluded infection by *S. carcini* had no overall effect on the molt increments of *Pachygrapsus marmoratus.* However, a few extremely feminized male *P. marmoratus* did have relatively small molt increments. In the present study, infection of *Pugettia producta* by *H. californicus* had no significant effect on molt increments of immature molts (Fig. 2) nor molts of puberty (Fig. 3). These molt increment values also agreed well with those measured by Born (1970) and Hines (pers. comm.) for unparasitized *P. producta*. The molt of puberty for *P. producta* was the terminal ecdysis, during which the parasite would have been most developed and presumably would have been making the largest energy demands upon the host. The lack of a significant difference between increments of immature molts at the molt of puberty coupled with no observed differences in instar duration between parasitized and unparasitized crabs (Fig. 4) justified excluding differential juvenile growth as an explanation of *P. producta* size differences.

The size of parasitized crabs could have been determined by an environmental factor and not the parasite. If season of recruitment or algal substrate (food) influenced *Pugettia* size and if *Heterosaccus* larvae were seasonal or restricted spatially within the habitat, it was possible that only crabs that were going to be small adults had been exposed to infection. Because no difference was detected in instar duration (Fig. 4), I assumed that similar-sized parasitized and unparasitized crabs had recruited concurrently. By following growth of similar-sized parasitized and unparasitized crabs from the same microhabitat, a mutually exclusive experiment was conducted. If all the crabs had been observed to pass through the same number of instars before maturing, the existence of an independent factor determining parasitized host size would have been supported. However, parasitized crabs underwent significantly fewer ecdyses before maturation (Table II). This result supported the precocious maturity explanation while rejecting differences in spatial or temporal variability of parasitization as the cause of differential size. Field data also supported the precocious maturity explanation. Parasitized crabs were found undergoing molts of puberty in far greater relative numbers than unparasitized crabs at the beginning of the maturation season (Fig. 6). In May 70% of parasitized crabs above 30 mm carapace length molted to maturity when they molted, a level not attained by the unparasitized population until 3 months later. Consequently, the simplest explanation for the smaller adult size of parasitized *P. producta* was that parasitized crabs passed through fewer instars before the molt of puberty.

Previous explanations of variation in adult size of *Pugettia*, readily incorporated the effect of the rhizocephalan on *Pugettia* size. Born (1970) felt that the broad size range of adult *P. producta* was a result of recruitment through the year with seasonal maturation. Crabs that did not mature one season would continue to grow and become the largest crabs a year later. Kuris (1971) working with the shore crab, *Hemigrapsus oregonensis*, broadened the model, postulating a size-threshold above which size juvenile crabs would undergo the molt of puberty. The size-threshold could vary during the year. Seasonality of the molt of puberty resulted from a seasonal decrease in this size-threshold. Crabs within size-classes between the two thresholds complete the molt of puberty after the threshold falls. Kuris (1971) did not state which conditions might cause such a decrease, but suggested that a seasonal increase in the size-threshold for maturation might be adaptive. Relatively small *H. oregonensis* matured at a time (the winter) when growth conditions were poor but reproduction was high. Inclusion of a stochastic element into the Born-Kuris model can explain the observation (Hartnoll, 1963, p. 451; Born, 1970; O'Brien, this paper, Table II) that not all majid crabs pass through the molt of puberty together even if they are the same size and are molting concurrently. Since not all crabs larger than the size-threshold undergo the molt of puberty at the very next ecdysis, I suggest that it is the probability of undergoing the molt of puberty that increases with crab size.

The essentials of the size-threshold model were supported by the data from the Coal Oil Point population. Precocious maturity can be explained as a greater sensitivity of parasitized crabs to the stimuli that induce maturation above the size-threshold.

Since the minimum sizes of parasitized and unparasitized *Pugettia* were equivalent (Fig. 1), the value of the size-threshold was not affected by the rhizocephalan. In fact, early in the maturation season (May and June), the mean size of newly matured animals did not differ between parasitized and unparasitized crabs (Table III). The size-threshold of maturation appeared to have suddenly lowered before May and June at Coal Oil Point (Fig. 7C, D). Less than half of the unparasitized juveniles that molted during the early maturation season (May and June) underwent a molt of puberty (Fig. 6), thus, mean size of the cohort of unparasitized juveniles continued to increase to the middle of the maturation season (July and August) (Fig. 7E). While small unparasitized adults matured throughout the maturation season as shown by the equivalence of the lower size-ranges, the upper range and mean size steadily increased which meant that large adults appeared during the middle and late maturation season (July through October) (Table III, Figs. 7E, G).

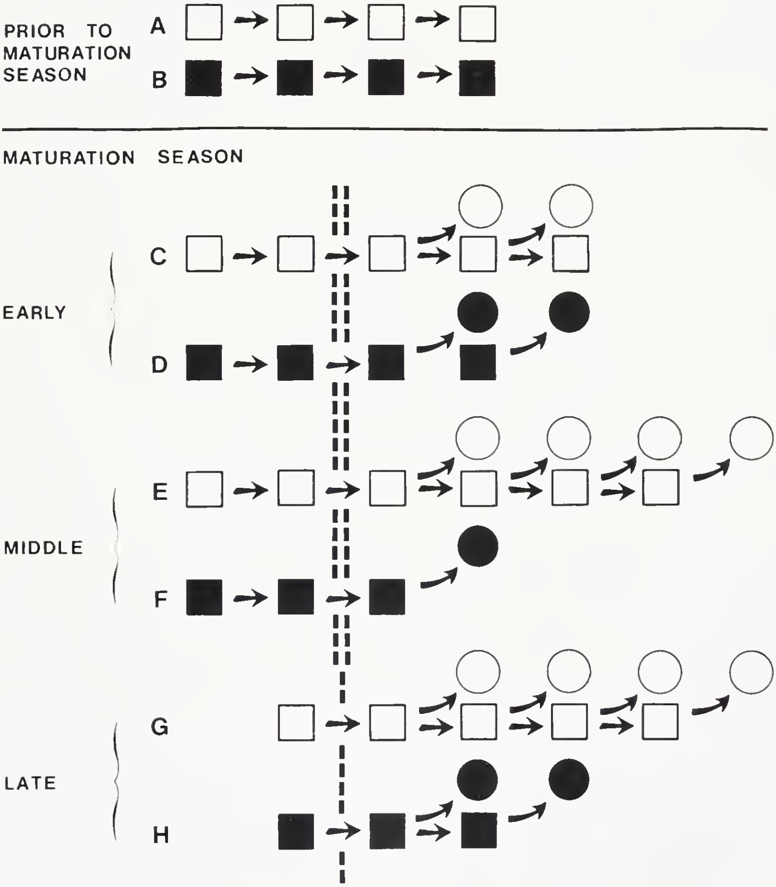

FIGURE 7. Schematic representation of a model explaining differences in adult size between unparasitized *P. producta* and those parasitized by *H. californicus* based on the Born-Kuris size-threshold model. Representations are as follows: circles = adult crabs, squares = juvenile crabs, diagonal arrows = molts of puberty, and horizontal arrows = immature molts. Absence of arrows between symbols indicates individuals are not passing through those particular instars at that time. Size increases from left to right with vertically aligned symbols representing individuals of equivalent size. The position and number of the broken lines indicate initial size and relative intensity of the size-threshold effect. See text for details.

The timing of maturation of parasitized hosts was much different than that of unparasitized crabs. A significantly higher percentage of parasitized juveniles that molted in May and June underwent the molt of puberty than did unparasitized juveniles (Fig. 6). In other words, few parasitized crabs above the size-threshold underwent immature molts (Fig. 7D) while large numbers of unparasitized juveniles continued to do so (Fig. 7C). There was no significant difference between mean size of newly matured parasitized and unparasitized crabs in May and June (Table III), because equivalent growth rates of juveniles (Figs. 2, 3, and 4) resulted in equivalent size of the juvenile cohorts from which the adults arose (Figs. 7A, B, C, and D). However, the lack of immature molts by parasitized juveniles early in the maturation season implied that the cohort of parasitized juveniles did not increase in size (Fig. 7F) and, consequently, there were no new mature hosts larger than 63 mm in the middle of the maturation season (July and August) (Table III). Parasitized hosts underwent the molt of puberty immediately following attainment of the size-threshold as evidenced by the relative pubertal molt rate close to 100% (Fig. 6). This explains why the smallest mode of parasitized *P. producta* contained more animals than modes in the larger parasitized size-classes (Fig. 1).

The conditions that induced the molt of puberty appeared to wane in the later maturation season (September and October). The pubertal molt rate for parasitized crabs decreased in September (Fig. 6) and mean size of newly matured parasitized hosts increased slightly, but still remained significantly less than mean size of unparasitized animals (Table III, Figs. 7G, H). Events late in the maturation season did not influence population structure of parasitized hosts as much as earlier maturations because the number of crabs maturing during this time was relatively low (Fig. 5). It was the cessation of the immature growth phase by parasitized crabs in the middle months of the maturation season (Fig. 7F) that resulted in the smaller mean size of *P. producta* parasitized by *H. californicus.*

In the *Heterosaccus-Pugettia* system, laboratory observations did not detect any differences between the two most commonly measured parameters of crustacean growth, molt increment, and instar duration. Instead, field observations and the laboratory growth experiment supported an explanation of precocious maturity for the observed differences in size between parasitized and unparasitized adults. The average parasitized adult crab passed through fewer instars than the average unparasitized adult. Thus, the difference in size between two groups of crabs was not attributable to a difference in the growth rate, but to a difference in the duration of the juvenile growth phase. Variation in the number of juvenile instars is probably not limited to parasitic castrator-crustacean host systems, rather such variation is simply more easily observed within such systems. Any thorough investigation into the causes of size differences among groups of adult crustaceans must consider variation in the number of juvenile instars as one of the reasonable explanations to be tested.

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AN EPITHELIAL CELL-FREE PREPARATION OF THE MOTOR NERVE NET OF *CYANEA* (COELENTERATA; SCYPHOZOA)

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ABSTRACT

Neurons of the motor nerve net of the jellyfish *Cyanea* lie between the cell bodies and muscle tails of myoepithelial cells. These myoepithelial cells are connected by septate desmosomes and, consequently, constitute a diffusion barrier and limit access to the neurons. Two techniques have been developed to remove the myoepithelial cells. One employs osmotic shock, the other, oxidation of the surface of the myoepithelial cells. The neurons remain in place and attach to the underlying mesoglea. Individual neurons retain their normal appearance and, when bathed in a saline that matches the free-ion content of the mesoglea, they retain their resting potentials and produce normal action potentials. Furthermore, the synapses continue to function normally. By supplementing the saline, these preparations can be maintained in good condition for several days. Under these culture conditions, neurons damaged during isolation produce growth cones and appear to form connections with other neurons.

INTRODUCTION

The motor nerve net of the jellyfish *Cyanea capillata* is a network of bipolar neurons that transmits action potentials from the marginal ganglia to the swimming musculature (Anderson and Schwab, 1981). The neurons are connected by symmetrical chemical synapses (Anderson and Schwab, 1981), implying that conduction across the synapse is functionally non-polarized. Although the cells are small, the cell bodies are large enough (10–20 μm) to allow intracellular recordings. Estimates of the cable constants of the neurons and the arrangement of the synapses indicate that an electrode in the cell body of a neuron will record all that occurs at the terminal (Anderson and Schwab, 1983). Consequently, this preparation can provide information about the physiology of chemical synapses in this animal and, because of the physiological conventionality of these neurons (Anderson and Schwab, 1983), synapses in general.

The preparation used is the peri-rhopalial tissue (Anderson and Schwab, 1981). Here, neurons of the motor nerve net lie between the cell bodies and muscle tails of the myoepithelium that forms the ectoderm of the peri-rhopalial tissue (Anderson and Schwab, 1981). At their outer boundaries these myoepithelial cells are joined by septate desmosomes (Anderson and Schwab, 1981) suggesting they present a diffusion barrier for drugs and putative transmitters, as well as physically limit access to the neurons and their synapses. For these reasons, it would be useful to be able to remove these overlying cells. Two techniques for doing so have been developed and are described here. The first employs a combination of osmotic shock and Ca^{++}/Mg^{++}-free sea water, while the second relies on the oxidation of the surface of the myoepithelial

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cells by sodium hypochlorite. Both techniques were successful although the latter proved to be more reliable and useful.

Early studies with the exposed neurons revealed that their resting potentials and excitability were altered when they were bathed in sea water, the usual saline for the earlier experiments with unexposed neurons. This observation prompted an investigation into the true ionic environment of the neurons, since bathing the exposed cells in sea water might, on its own, account for their altered responses. There are no obvious diffusion barriers between the neurons and mesoglea of the animal (Anderson and Schwab, 1981). Assuming that the free ion content of the mesoglea would be similar, if not identical to that of the extracellular fluid, the mesoglea was used as the source of fluid for ion analysis. Exposed neurons bathed in the resulting saline had normal resting potentials and excitability. Furthermore, by supplementing the saline these preparations could be maintained in good condition for several days.

MATERIALS AND METHODS

Ion analysis

Specimens of *Cyanea capillata* with bell diameters of 10 cm or greater were collected in the vicinity of the C. V. Whitney Laboratory. Shortly after collection, an animal was removed from the holding tank and its tentacles and oral veils removed. The central part of the bell was excised and cut into 1 to 2 cm cubes and adhering tissue and epithelial layers removed. The cleaned, now completely transparent, pieces of mesoglea were rinsed briefly in distilled water, blotted dry on filter paper, and placed in an ultra-filtration cell (Amicon 2100 CF 50), which was centrifuged for 15 min at 2500 rpm. This procedure produced approximately 2.5 ml of fluid per animal. The fluid from each animal was analyzed for osmotic strength (Wescor 5100B) and then divided into several separate vials and frozen.

Sodium and potassium concentrations were measured by flame photometry (Instrumentation Laboratory 443), calcium and magnesium concentrations by atomic absorption (Perkin-Elmer 2380), and chloride ion concentration by titration. For sulfate measurements, the fluid samples were first acid hydrolyzed to denature residual protein and then total sulfate was measured using $^{123}BaSO_4$ precipitation (Renfro and Dickman, 1980). In each case, fluid from three vials was analyzed for each animal and the final concentration calculated as the average of 5 animals (Table I).

The saline developed from this study was prepared according to the method of Wilkens (1970). Since the osmotic strength of the fluid from the mesoglea was found to be within the range of ambient sea water, all stock solutions were prepared isos-

TABLE I

The concentrations (mM) of ions in fluid from the mesoglea of Cyanea

Animal #	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	SO_4^{--}
Cy 1	397.4	16.5	9.8	31.0	575.7	5.28
Cy 2	409.5	14.3	9.3	29.2	586.7	3.74
Cy 3	377.0	13.0	9.5	27.6	535.3	3.34
Cy 4	389.4	11.3	9.4	29.4	553.7	7.19
Cy 5	374.3	11.8	9.4	27.9	513.3	8.69
Mean	389.5	13.4	9.5	20.0	552.9	5.65
S.E.M.	6.52	0.94	0.09	0.61	13.31	1.02

motically with ambient sea water. The final concentrations (mM) of the various salts in the saline were: NaCl, 390; KCl, 13.4; $CaCl_2$, 9.5; $MgCl_2$, 24; $MgSO_4$, 5; choline chloride, 41.5; $NaHCO_3$, 2.7. Choline chloride was added to elevate the chloride concentration to that measured by ion analysis. Ca^{++}/Mg^{++}-free saline was prepared by adding an equivalent amount of NaCl. Na^+-free saline was prepared using Tris-Cl. The pH of homogenized mesoglea was 7.4 and the pH of all salines was adjusted to this value. For long-term culture, the saline was supplemented with 1 $mg \cdot ml^{-1}$ glucose (Sigma), 2% Fetal Bovine Serum (GIBCO), and 1% penstrep with fungizone (GIBCO). No attempt was made to use sterile procedures during the production or maintenance of the preparations.

Electrophysiological techniques

The recording techniques employed here have been described in detail elsewhere (Anderson and Schwab, 1981; 1983). Briefly, pieces of peri-rhopalial tissue (Anderson and Schwab, 1981) were removed from the animal and pinned with cactus spines (*Opuntia* sp.) to a layer of Sylgard (Dow Corning) on the bottom of a plastic petri dish. When such preparations were examined using Modulation Contrast Optics, neurons of the motor nerve net were seen very clearly (Fig. 1A). This basic procedure was followed both for intact preparations and as the first step in the preparation of exposed cells, although, with the latter, the adjoining muscle bands were not removed. Preparations of exposed cells were bathed in *Cyanea* saline. Tetrodotoxin (TTX), saxitoxin (STX) and Ni^{++} ($NiCl_2 \cdot 6H_2O$) were added directly to the saline as needed.

For intracellular recordings cells were impaled with 3 M KCl-filled microelectrodes pulled from either thin-walled glass (Supertip, Frederick Haer) (10–20 MΩ impedance) or conventional thicker-walled capillary glass (WP Instruments) (40–60 MΩ). Action potentials were evoked by current injected through the recording electrode. Injected current was measured with a virtual ground circuit in the ground return circuit; a high-frequency filter in this circuit tended to round-off the square current pulses (see Fig. 3A). Signals were amplified using conventional capacity-compensated amplifiers (Dagan Instruments, Getting Instruments) and displayed on a digital oscilloscope (Nicolet Instruments). Hard copies of stored records were produced using a Houston Instruments 100 X-Y plotter.

Removal of the epithelium

Several procedures have been used, with various results, to expose the neurons. No attempt was made to use enzymatic techniques since these might produce subtle changes that could escape detection. Mechanical abrasion of the overlying myoepithelial cells with a microelectrode held in a micro-manipulator was effective in exposing the neurons, but was extremely time consuming.

The most effective and reliable techniques were based either on brief osmotic shock or brief oxidation of the myoepithelial cell surface. The former technique was used because the myoepithelial cells all contain a large vacuole that occupies most of their volume (Anderson and Schwab, 1981). It was felt that if these cells could be damaged selectively, by bursting their vacuoles, the muscle tails might contract and release the cells. This procedure worked well, but better results were obtained by subsequent treatment with Ca^{++}/Mg^{++}-free saline that contained 10 mM EDTA.

When these two treatments were combined the results were extremely good, but also extremely variable. Sometimes prolonged exposure (5 min or more) to 20% sea water, or even distilled water was necessary, while with other preparations 30 seconds in low strength sea water proved too long, and when the solutions were changed the entire ectoderm would float free. The same applied to the Ca^{++}/Mg^{++}-free treatments. Even in those preparations which were judged to have been appropriately treated, the ectoderm would be released from the mesoglea in a single sheet if subjected to excessive mechanical agitation. For this reason, mechanical disturbance was minimized; solutions were changed by pipette if necessary and the preparations left undisturbed when possible. Because of the variability encountered in this work, it is difficult to give a particular recipe for the most effective protocol. However, the basic procedure can be summarized as brief osmotic shock in 20% sea water until vacuole bursting is first observed, followed with one rinse by Ca^{++}/Mg^{++}-free saline with 10 m*M* EDTA. The preparation must be carefully examined during this latter treatment and at the first appearance of epithelial cell-free regions, the solution replaced with the final solution (supplemented *Cyanea* saline), with minimal disturbance. At this stage, the preparations were transferred to a refrigerator (9°C) for 12–24 hours, after which time the released epithelial cells would have formed a thin mucous-rich film over the entire surface. This layer would be removed with a pipette, leaving the neurons attached to the mesoglea.

For the second procedure, preparations of peri-rhopalial tissue were pinned out in the same manner and then immersed in a 0.05% solution of sodium hypochlorite (1:100 dilution of domestic bleach) for 1–5 seconds. This solution was poured off and the preparation immediately rinsed 5 times in saline and set aside, once again with minimal mechanical disturbance. After 2–3 hours the debris could be removed with a pipette, exposing the neurons. At this point the preparations were bathed in supplemented saline and kept at 9°C until needed.

Electron microscopy

Preparations of exposed neurons were fixed in 2.5% gluteraldehyde in 0.4 *M* Millonig's phosphate buffer and post-fixed in 1% phosphate buffered osmium. For ease of handling the pieces of fixed tissue were transferred to small pieces of Sylgard (Dow Corning, Michigan) and pinned in place using cactus spines (*Opuntia* sp.), before being dehydrated with ethanol, critically-point dried, and coated with gold/palladium. The tissue was examined using a JSM 35C operated at 15 kV.

RESULTS

The procedures used to expose the neurons had a wide range of effects, and the results were often variable. The osmotic technique sometimes produced preparations in which most, if not all, of the nerve net was exposed with only small amounts of epithelium remaining (Fig. 1B, C, E–G); on other occasions, the epithelium was seemingly unaffected by the procedure, even though the epithelial cells appeared to have been damaged and dislodged during EDTA treatment. Finally, with some preparations the entire epithelium disappeared with only a few neurons remaining (Fig. 1D, H); the remainder of the preparation was completely clear. This variability was due, in part, to differences between animals. If pieces of peri-rhopalial tissue from different animals were pinned in the same dish, and thereby given identical treatment, the results for the two preparations were often quite different.

With the bleach treatment, too, the results were variable, but here the epithelium was almost always immediately irreversibly damaged; the variability occurred when the resulting debris was removed. With some preparations the neurons remained attached to the mesoglea and the debris could be removed easily; with others, the debris could not be removed without taking the neurons too. Nevertheless, this technique was much more reliable and convenient. The epithelial cells could be removed

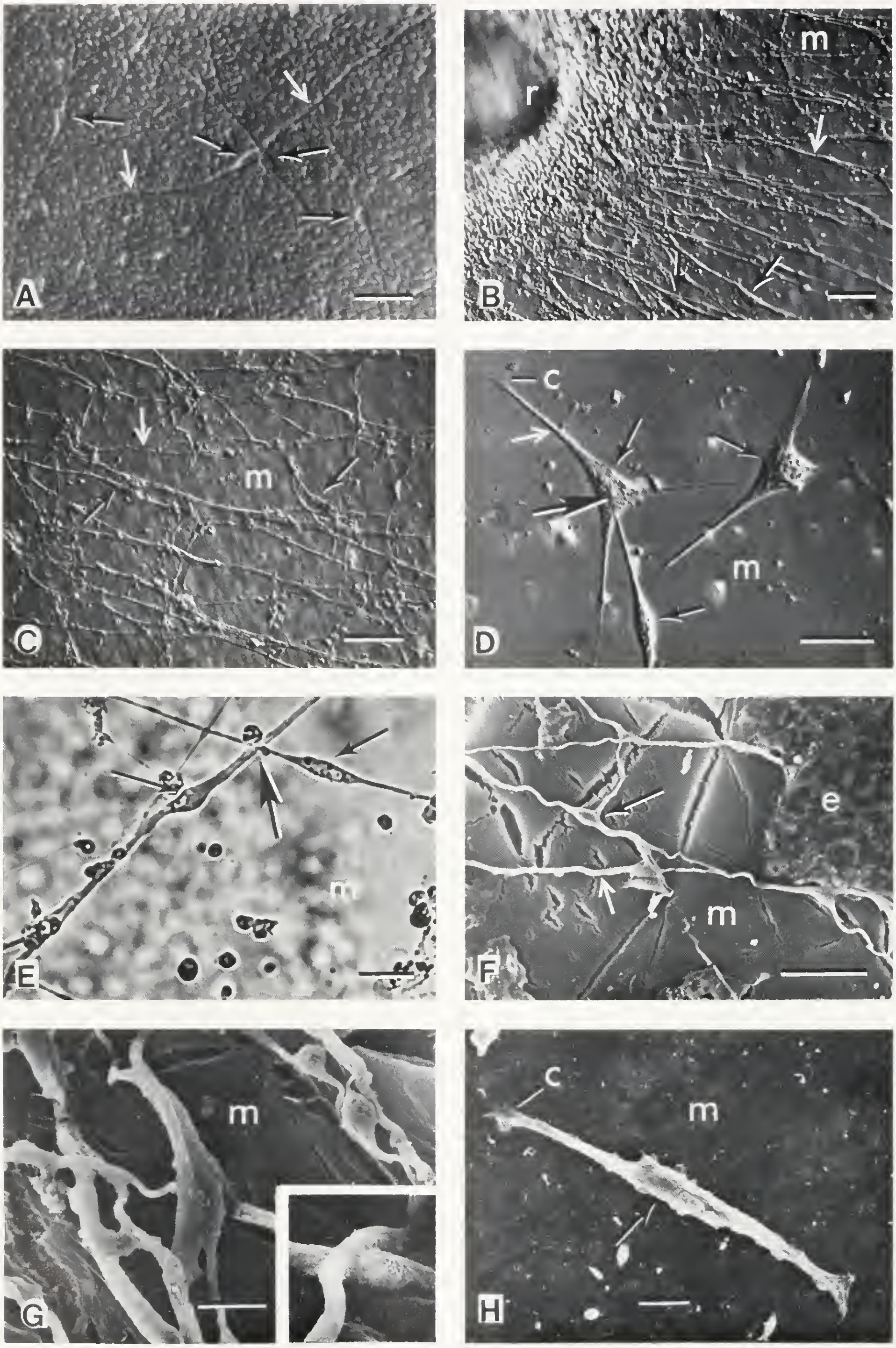

FIGURE 1. (A) A modulation contrast micrograph of the peri-rhopalial tissue of *Cyanea*. The cell bodies (black arrows) and axons (white arrows) of neurons of the motor nerve net are clearly visible. The cobblestone appearance of the bulk of the tissue is due to the vacuoles in the myoepithelial cells whose cell bodies cover the neurons. Bar = 50 μm. (B) A low-power modulation contrast micrograph of a portion of an epithelial cell-free preparation of the peri-rhopalial tissue immediately adjacent to the rhopalial (r).

more rapidly with the bleach treatment (2–3 hours as opposed to 12 hours), and subjective assessments of the end points of the stages in the osmotic protocol were avoided.

In preparations with a low neuron density, some cells were invariably found close to the muscle bands and in a fan-like array emerging from the rhopalium (Fig. 1B). This distribution may reflect the additional mechanical support given the neurons by these structures. If all muscle were removed from the peri-rhopalial tissue at the outset of the treatment, the stripping process occurred as described, but the preparation would later shrink and pull free from the cactus spines. For this reason, the usual preparation was one in which the adjoining radial and circular muscle bands were left intact. These preparations were less susceptible to shrinkage, although their marginal ends, which lacked muscle, would often pull away from the pins.

Morphology of exposed neurons

For the most part, the exposed cells retained their characteristic bipolar shape (Anderson and Schwab, 1981). This was particularly so for newly prepared preparations in which the density of neurons remained high (Fig. 1B, C). With such preparations individual neurons could be traced over considerable distances through several synapses. The dimensions of the neurons were no different from those of cells in intact preparations, although even with the cells so visible, it was not possible to determine the lengths of the longer fibers; the criss-crossing and merging of axons made it difficult to trace any one process for any distance. When preparations were maintained in supplemented saline for any length of time the dimensions and appearance of the neurons did change. Most noticeably, the cells flattened out onto the mesoglea, and as a result appeared far larger (Fig. 1D); many cells produced supernumerary processes (Fig. 1D).

In many exposed neurons, intracellular organelles, such as the nucleus, nucleolus, and a variety of vesicular structures, could be seen clearly (Fig. 1D, E) and movement of the organelles often could be discerned. Scanning electron microscopy of the exposed neurons revealed that their surfaces were, for the most part, clear of adhering material (Fig. 1F–H) and individual axons could be traced over considerable distances.

In preparations in which only a few neurons remained, the axons of the exposed cells were usually very short; the total length of the cell could be as little as 150 μm,

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The motor nerve net neurons remain in place and lie on the surface of the transparent mesoglea (m). Individual nerve cell bodies (black arrows) and their axons (white arrows) are visible. Bar = 100 μm. (C) A low-power modulation contrast micrograph of exposed neurons. Individual axons (white arrow) and cell bodies (black arrows) are visible and processes of the cells cross one another to form a complex two-dimensional nerve net: m—mesoglea. Bar = 100 μm. (D) A modulation contrast micrograph of neurons in a preparation in which the density of neurons was low. This preparation had been maintained for 1–2 days as evidenced by the flattening of the neurons, the formation of growth cones (c), and the unusually high frequency of tripolar neurons. The processes from the neurons make contact with one another (large arrows) at discrete points rather than in the normal *en passant* fashion. Numerous small vesicular structures can be seen in these cells. Many such organelles were motile. Bar = 50 μm. (E) A phase micrograph of a presumed synapse (large arrow) between two neurons in an epithelial cell-free preparation. The neurons (small arrow) are obvious against the clear mesoglea (m). Bar = 25 μm. (F) A low-power scanning electron micrograph of an epithelial cell-free preparation. At the edge of the exposed area, the surface of the epithelial cell layer (e) is visible. The axons (white arrows) and cell bodies (black arrows) of exposed neurons are visible against the mesoglea (m) which had fractured during drying. Bar = 50 μm. (G) A higher-power scanning micrograph of exposed neurons. The axons of the neurons criss-cross and parallel one another to form a complex series of connections: m—mesoglea. Bar = 20 μm. Inset. A presumed synapse between two neurons. The axons are very closely opposed and no gap is visible between them. (H) A scanning micrograph of a single exposed neuron. The soma is visible as a swelling (arrow) but the axons are very short. At one end the cut axon forms a growth cone (c): m—mesoglea. Bar = 20 μm.

as opposed to the 0.5–1.0 cm that has been reported for cells in other medusae (Horridge, 1954). When these preparations were maintained, the neurons developed additional processes as already described, and the cut ends of the axons produced growth cones (Figs. 1D, H; 2) which migrated across the mesoglea. Fine filopodia (0.13–0.4 μm diameter) projected from the expanded axon tip. The lengths of the filopodia were variable, but several could be traced for 30–40 μm. The cone itself could be up ~20 μm in diameter and much of this consisted of a thin sheet or lamellipodium.

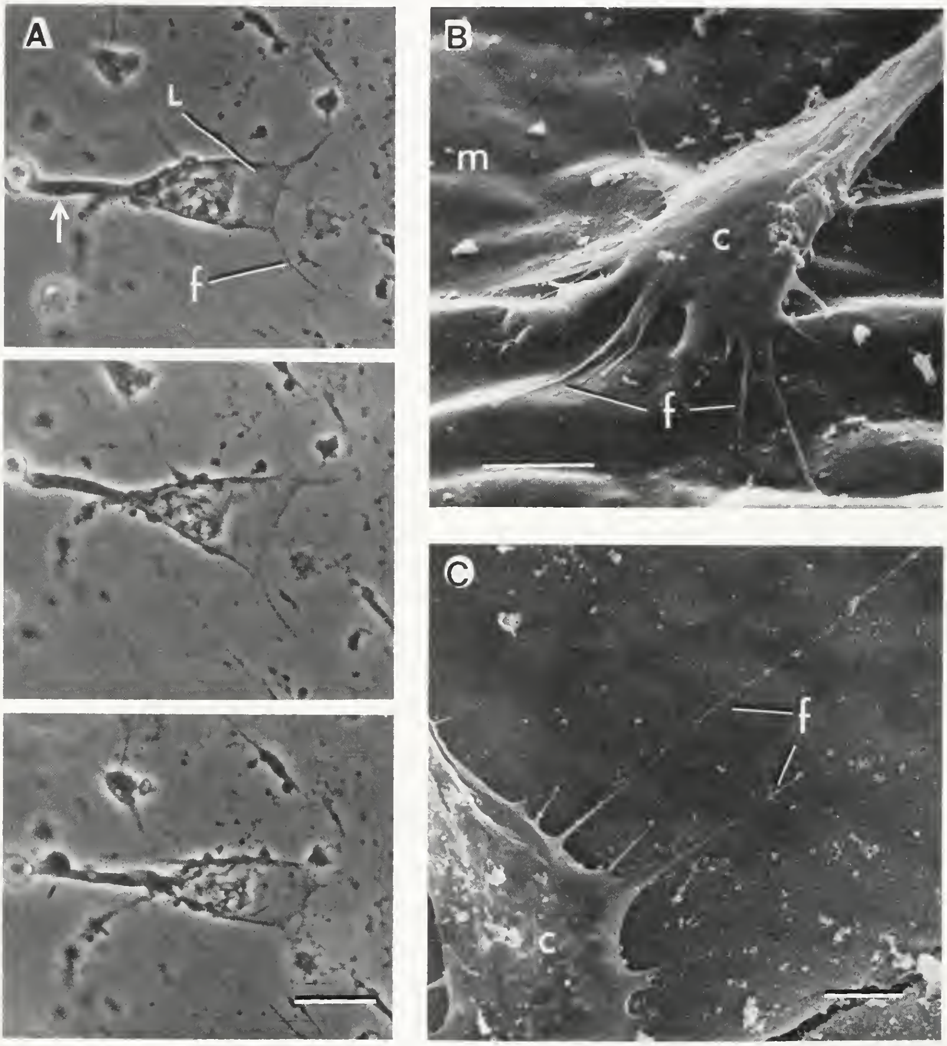

FIGURE 2. (A) Three phase micrographs of single growth cone taken at intervals of 8 and 17 minutes. The growth cone is an expansion at the end of the axon (white arrow) and consists of a broad lamelipodium (L) from which emerge numerous filopodia (f). Growth cones continually changed shape as they spread over the mesoglea. Such shape changes are obvious here. Bar = 20 μm. (B & C) Scanning electron micrographs of growth cones (c) on exposed neurons. Filopodia (f) emerge from the expanded cone and extend for long distances over the mesoglea (m). Bar = 5 μm.

The cones were extremely active: they changed their shape continuously and rapidly as the filopodia extended and retracted from the cone. Figure 2A shows three micrographs of the same cone taken at intervals of 8 and 17 minutes. The overall shape of the cone changed throughout the series, as did its position relative to other objects in the field of view. No attempt was made to quantify the rate of growth of the axon or filopodia. With preparations that contained fairly large numbers of neurons, the growing axons did appear to form contacts with other neurons (Fig. 1D) but, at this time, it is not clear if those connections were functional synapses.

Intracellular recordings from exposed neurons

Intracellular recording from unexposed neurons is quite feasible, but fairly difficult since the cells are very small. The overlying epithelium may aid impalement by preventing lateral movements of the neurons, but when the epithelium is present a microelectrode can be used for only one attempted penetration. If that is unsuccessful, then the microelectrode has to be discarded. This need to constantly change the electrodes is obviously time-consuming. With exposed cells, the problems are greatly reduced. It is easier to impale the exposed cells, and a single microelectrode can be used for several successive cells.

When exposed cells were bathed in normal sea water or artificial sea water (ASW), their resting potentials were significantly different from those of cells covered by intact epithelium (*in situ* cells). The mean resting potential of *in situ* cells was −58 ± 2 mV (SEM; n = 40) (Anderson and Schwab, 1983), while that of exposed cells was −67.9 ± 0.9 mV (n = 26). Furthermore, it proved very difficult to evoke action potentials in exposed cells. Extracellular axonal stimulation, usually the most effective means of evoking action potentials in these cells, was successful only when high intensity stimuli were used, and usually the axon under the stimulating electrode was so damaged by the stimulus that further spikes could not be evoked without moving the stimulating electrode. Intracellular current injection, a technique frequently successful with *in situ* cells, was almost totally ineffective. It was these findings that prompted the search for a suitable saline for these neurons.

Exposed cells bathed in *Cyanea* saline had resting potentials in the range of −46 to −69 mV (mean = −58.5 ± 1.5; n = 16). These values are almost identical to those of *in situ* cells. Furthermore, their excitability remained unaffected. Representative action potentials from exposed cells are included in Figure 3. These spikes were evoked by intracellular current injection and they display much of the variability and complexity of spikes recorded from neurons in intact preparations (Anderson and Schwab, 1983). The mean overshoot of action potentials in exposed cells bathed in saline that contained 10 m*M* Ni^{++} to block the synapses was 38 mV ± 3.4 (SEM; n = 6). This compares favorably with the 33 mV overshoot for *in situ* cells under similar conditions. The hyperpolarizing undershoot in the exposed cell took up to 1850 ms to decay following a single action potential. Once again, this is consistent with results from *in situ* cells (Anderson and Schwab, 1983). Superimposed on the action potentials were small depolarizing potentials. These have been shown to be synaptic potentials (Anderson and Schwab, 1983). With some cells, these occurred up to 28 ms after the spike (Fig. 3B). This delay is far larger than that typically encountered with *in situ* neurons and may, in part, reflect damage. Alternatively, the increased delay may merely reflect the added time taken for an impulse to spread through the less dense nerve net, where pathways would be longer, and return to the recorded cell via the recurrent pathways inevitably present in a diffuse nerve net (Anderson and Schwab, 1983).

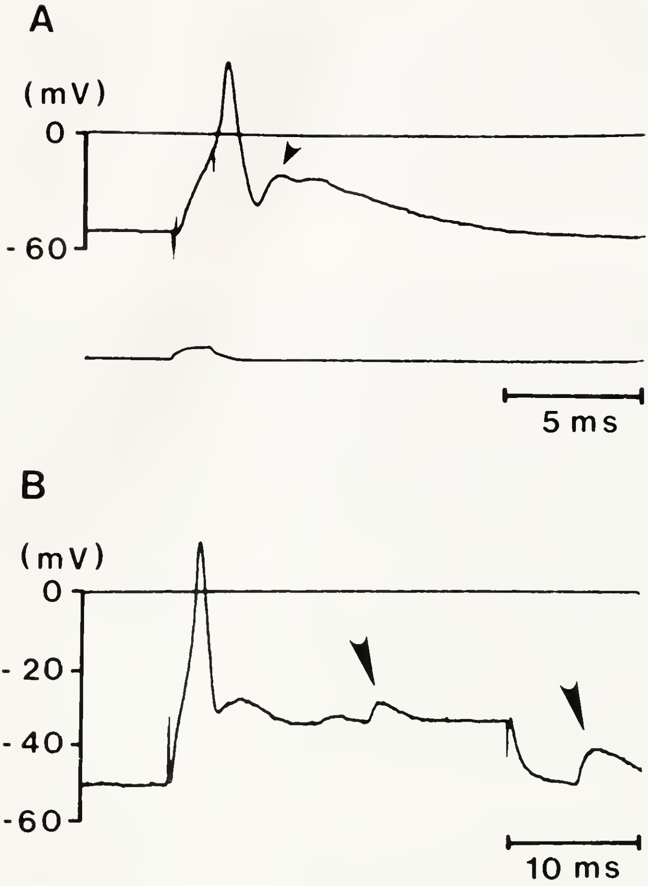

FIGURE 3. Action potentials recorded intracellularly from exposed neurons. (A) Upper trace: a single action potential evoked by a brief (2 ms) current pulse. Lower trace: current record. The action potential occurred when the current pulse had ended and was followed by a complex synaptic depolarization (arrow head). (B) A single action potential evoked by a longer current pulse. Individual synaptic potentials (arrowheads) are visible.

To further verify that the exposed cells were in good condition, they were current clamped, and I/V curves produced (Fig. 4). As with *in situ* cells, they displayed pronounced delayed rectification and, for hyperpolarizing pulses, had input impedances in the range 35–75 MΩ. This is consistent with data from *in situ* neurons (Anderson and Schwab, unpub.).

Preparations of exposed neurons maintained at 9°C in supplemented *Cyanea* saline remained active and functional for several days. When these preparations included swimming muscle, a stimulus to the center of the preparation would produce muscle contraction if there were an intact pathway to the muscle. This phenomenon occurred for up to 4 days post-exposure, and action potentials in cells maintained for this period contained synaptic potentials typical of those in freshly exposed or *in situ* cells.

One of the chief reasons for developing the exposed cell preparation was to remove the permeability barrier posed by the overlying epithelial cell bodies. Earlier (Anderson

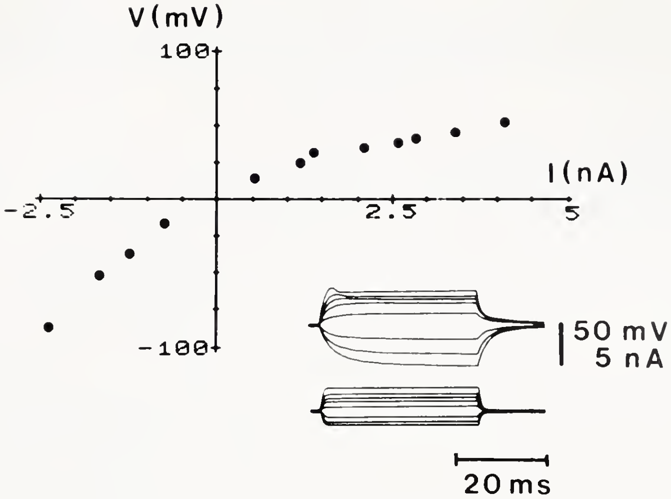

FIGURE 4. The current/voltage relationships of an exposed cell. Current clamp records from this cell are included as an inset. The exposed neurons have input impedances typical of those of unexposed neurons and display delayed rectification upon depolarization.

and Schwab, 1983) it was noted that the sodium channel blocker tetrodotoxin (TTX) (Narahashi *et al.*, 1964) was ineffective, even at high concentrations. It could be argued, however, that this observation was the result of impermeability, rather than true TTX insensitivity. However, exposed cells continued to produce normal action potentials after 24 hours in 1.25×10^{-5} *M* TTX. The cells were also insensitive to 10^{-6} *M* saxitoxin (STX), which had not been used previously with these or other coelenterate neurons. Since the effects of TTX and STX are similar if not identical (Narahashi, 1974) the absence of STX sensitivity is not surprising.

The amplitude of the action potential in intact preparations is dependent on the extracellular sodium concentration (Anderson and Schwab, 1983). However, the relationship between the two (88 mV/decade) was not that predicted by the Nernst equation, and at the time, it was thought that the discrepancy might be a consequence of ion pumps in the overlying epithelium. To test for this, these experiments were repeated using exposed neurons.

Preparations were bathed in *Cyanea* saline that contained either the normal amount of sodium or 50% sodium (Tris substituted). EPSPs, which confer amplitude and waveform variability to the spike, were blocked by the addition of 10 m*M* Ni^{++} to the medium. Action potentials were evoked by current injected through the microelectrode. The results confirmed the earlier finding that the spike is sodium dependent (Anderson and Schwab, 1983). The relationship between overshoot amplitude and extracellular sodium was 49.7 mV/decade. Calcium-free saline was not used here because of its potential for artificially depolarizing the neurons (Frankenheuser and Hodgkin, 1957), and although the Ca^{++} channels were blocked by Ni^{++} (Adams *et al.*, 1980), some Ca^{++} may have entered through the Na^{+} channels. This Ca^{++} entry could account for the discrepancy between the slope obtained here, and that predicted by the Nernst equation (58 mV/decade). Interestingly, the slope obtained here is similar to that for *in situ* cells in the presence of ouabain (47 mV/decade) (Anderson and Schwab, unpub.), confirming the earlier belief (Anderson and Schwab, 1983) that the unusual relationship between spike overshoot in *in situ* cells and external Na^+ concentration can be explained by the action of transepithelial ion pumps.

DISCUSSION

The neurons of the motor nerve net of *Cyanea* are normally covered by the cell bodies of the myoepithelial cells of the peri-rhopalial tissue ectoderm (Anderson and Schwab, 1981). While these cells do not obscure the neurons (Fig. 1A) nor prevent cell impalement, they form an obvious barrier that restricts physical access to the neurons and, more important, to their synapses. This barrier would limit the usefulness of this preparation by restricting access of putative transmitters applied to the synapses. Here we describe the results of a study designed to circumvent these problems by devising ways of removing the overlying cells.

Two techniques are described. Although their effects were variable, both were very successful, producing good preparations of exposed, clean neurons. Of the two, the sodium hypochlorite technique was by far the more convenient and reliable. It is, however, the one most open to criticism. Sodium hypochlorite is a powerful oxidizer and one which, even in very low concentrations, could damage the neurons. However, it was used here assuming that the septate desmosomes that connect the myoepithelial cells would protect the underlying neurons, at least temporarily, by preventing the diffusion of the hypochlorite into the extracellular spaces. The fact that the physiology of the exposed neurons was seemingly normal supports this belief.

Neurons in the exposed cell preparations were morphologically normal and, when damaged, produced growth cones which closely resembled growth cones of higher organisms (Landis, 1983). The cut axons grew and appeared to form connections with other cells (Fig. 1D). The exposed neurons lie on the mesoglea, an acellular matrix with some chemical similarities with the basement membranes of vertebrates (Barzansky and Lenhoff, 1974; Barzansky *et al.*, 1975). Mesoglea is known to be an excellent matrix for attachment and spreading of coelenterate cells (Day and Lenhoff, 1981). Those authors used *Hydra* mesoglea as a substrate for attachment of a variety of non-specified *Hydra* cell types and found that of the various alternative substrates tested, including collagen and other tissue culture substrates, mesoglea was the most effective. It is not surprising, therefore, to find that *Cyanea* mesoglea is an excellent substrate for neurons from this species. It should be noted, however, that in intact peri-rhopalial tissue the neurons are separated from the mesoglea by the muscle tails of the myoepithelial cells (Anderson and Schwab, 1981) and have to settle onto the mesoglea once the intervening muscle tails have been removed. The fact that there is initially this separation between the mesoglea and the neurons explains why the newly prepared exposed cell preparation is so susceptible to mechanical disturbance.

This ability for the neurons to grow and apparently reform connections is not surprising. Romanes (1885) noted that when the subumbrella surface of *Aurelia* was cut with a shallow incision extending across the entire subumbrella, coordination of the muscles on either side of the cut was lost initially, but redeveloped after as little as 4 hours. This observation was interpreted as evidence for regeneration, a likely interpretation in view of the findings presented here.

When exposed neurons were bathed in sea water, their resting potentials and excitability were altered; specifically, the resting potentials were significantly more negative and the cells were far less excitable. These changes can be explained by differences between the ionic composition of the sea water (or ASW) and that of the extracellular fluid (Table II). The K^+ concentration of the extracellular fluid is higher than that of sea water, so exposed cells bathed in sea water would be hyperpolarized

TABLE II

Concentration (mM) of ions in fluid from Cyanea *and artificial sea water (ASW)*

	Na$^+$	K$^+$	Ca^{++}	Mg^{++}	Cl$^-$	SO$_4^{--}$
Cyanea c.	389.5	13.4	9.5	28.8	552.9	5.65
Cyanea c.[1]	442.0	16.2	19.9	100.0	556.0	22.4
ASW[2]	462.3	9.72	9.86	51.2	506.12	27.0

[1] From Koizumi and Hosoi (1936).
[2] From Wilkens (1970).

by the reduced K$^+$ concentration. Similarly, the Mg^{++} concentration of the mesogleal fluid is significantly lower than that of sea water, so when exposed cells were bathed in sea water they would be in an abnormally high extracellular Mg^{++} concentration. In amphibians, when the extracellular concentrations of divalent cations such as Ca^{++} and Ni^{++} are increased, frog nerve becomes less excitable and greater levels of depolarization are needed to elicit a given response (Hille, 1968). A similar situation could apply here when Mg^{++} levels are increased.

The only other remarkable difference between the ion contents of the mesoglea and sea water concerns sulfate. The sulfate concentration of the mesoglea was invariably less than that of sea water and showed the most variation. Neither of these findings is surprising. Denton and Shaw (1961) and later Mackay (1969), demonstrated that many gelatinous marine organisms, including coelenterates, regulate their buoyancy by altering their internal sulfate concentrations. Lift is obtained by exclusion of sulfate and, presumably, negative buoyancy by its accumulation. The jellyfish used in this study were collected by dipping from the surface and such animals would, according to this model, have achieved this position in the water column by excluding sulfate. The variability in the sulfate levels can be explained by a similar argument, considering that some animals were collected at a slightly greater depth than others, and would presumably be neutrally buoyant, hence having higher sulfate levels.

With the exception of these differences, the ionic composition of the mesoglea is essentially the same as that determined by Wilkins (1970) for sea water and similar to that measured in an earlier study (Koizumi and Hosoi, 1936) (Table II). It should be noted that this latter study used an intact piece of exumbrella, so some intracellular ions also would have been included. This and the less exact procedures used could account for the slight discrepancies between our findings and theirs.

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ALANINE UPTAKE BY ISOLATED ZOOXANTHELLAE OF THE MANGROVE JELLYFISH, *CASSIOPEA XAMACHANA*. I. TRANSPORT MECHANISMS AND UTILIZATION

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ABSTRACT

Freshly isolated zooxanthellae of the mangrove jellyfish *Cassiopea xamachana* were found to take up exogenous ^{14}C-alanine and incorporate the radiolabel into various cellular components. Transport was highly Na$^+$-dependent and lacked stereospecificity. Competition experiments with several L-amino acids and alanine analogs exhibited varying degrees of competitive inhibition, and these results are discussed in relation to the well described neutral amino acid transport systems known for many eukaryotic cells. Inhibition of uptake by KCN and N-ethylmaleimide indicated an active process and a significant electrogenic component to the energetics of uptake. Uptake of alanine was not affected by the inhibition of photosynthesis.

INTRODUCTION

Unicellular algae, termed zooxanthellae, occur in symbiotic relationships with a wide variety of marine invertebrates. Most of these associations occur in members of the phylum Cnidaria (Coelenterata), especially in hermatypic corals and sea anemones. Though the association is not an obligate one, the widespread occurrence and stability of such symbioses suggest that the metabolic relationships which exist between symbionts are well regulated and of mutual physiological advantage.

It is now well documented that the photosynthate produced by zooxanthellae can be translocated to animal host cells (for reviews, see Muscatine, 1974, 1980). In coral, these products are primarily glycerol with smaller amounts of glucose and alanine (Muscatine and Cernichiari, 1969; Lewis and Smith, 1971; Trench, 1974). Other studies on cnidarians suggest that significant amounts of lipid may also be translocated (Patton *et al.,* 1977; Blanquet *et al.,* 1979).

The quantity of organic material supplied to the animal may be considerable. von Holt and von Holt (1968) reported that in *Zoanthus* and *Condylactis* as much as 70% of the algal photosynthate may be transported in three hours. Trench (1971) found that about half of the radiolabeled carbon fixed by zooxanthellae of the sea anemone *Anthopleura* was transferred. Muscatine *et al.* (1981) recently estimated that the zooxanthellae of *Fungia* and *Pocillopora* can supply 63–69% of the daily respiratory carbon demand in these corals.

The back-transfer and utilization of animal metabolites by zooxanthellae, on the other hand, are not as well studied. Although Muscatine (1978) examined the uptake and retention of dissolved, inorganic nitrogen, little is known about the exchange of small organic molecules. Cook (1971) demonstrated the incorporation of isotope in the zooxanthellae of the anemone *Aiptasia* which had been fed ^{35}S-labeled mouse

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liver and suggested that the algae could exchange amino acids with host amino acid pools. Trench (1979) reported that when organic-^{35}S was fed to *Anthopleura,* up to 40% of the label was transferred from the anemone to the zooxanthellae. Thorington and Margulis (1981) also suggested that in green hydra, zoochlorellae and host compete for the available pool of organic compounds obtained from host feeding. They found that, with time, levels of radiolabeled metabolites decreased in the host as label increased in the algae. When hydra were maintained in the dark, algal cells could survive for months in the absence of photosynthesis. Thus, the ability to utilize host metabolites may considerably enhance the potential for algal survival and assure the stability of the association.

No investigations, however, have focused on the specific mechanisms by which such back-transfer occurs for any type of organic compound. This study was thus designed to characterize some aspects of amino acid uptake by freshly isolated zooxanthellae from the mangrove jellyfish *Cassiopea xamachana* and to compare these to the amino acid uptake mechanisms of other cell types. Alanine was chosen as a probe, since it is a common constituent of the free amino acid pools of eukaryotic cells, and characteristics of its uptake have been well studied. In addition, alanine has been shown to be an end-product of anaerobic metabolism in cnidarians (Ellington, 1977, 1980, and 1982), and thus may be produced by *Cassiopea* under the hypoxic conditions of the marine mangrove swamps which it inhabits.

Materials and Methods

Cassiopea xamachana were obtained from Marine Specimens Unlimited (Summerland Key, Florida) and maintained in the laboratory in filtered sea water (FSW) made from INSTANT OCEAN sea salts (Aquarium Systems, Eastlake, Ohio). All media were adjusted to pH 8.3 and a salinity of 29 ± 1 ppt. Animals were used within ten days of arrival.

For studies on sodium-specificity of amino acid uptake, artificial sea water and sodium-free medium were prepared according to the Woods Hole Formula (Cavanaugh, 1964). For sodium-free medium, appropriate amounts of choline chloride and $KHCO_3$, were substituted to maintain the same molarity of NaCl and $NaHC0_3$, respectively.

Isolation of zooxanthellae

Cassiopea zooxanthellae were freshly isolated for each experiment according to the methods of Blanquet *et al.* (1979). Final cell suspensions were checked for purity by light microscopy and standardized for all experiments at $3 \cdot 10^6$ cells ml^{-1}. Cell counts were determined by a hemocytometer. Levels of total chlorophyll, (chl a and c_2), expressed in μg chl ml^{-1}, were determined by the methods of Jeffrey and Haxo (1968). All experiments were performed under standard conditions of light (45 ± 3 μEinstein m^{-2} s^{-1}) and temperature (23 ± 2°C).

Radioisotope uptake studies

Chemicals. Radiolabeled L-(1-^{14}C)alanine (56.2 mCi mmole^{-1}), D-(1-^{14}C)alanine (56.2 mCi mmole^{-1}), L-(U-^{14}C)alanine (144 mCi mmole^{-1}), and (1-^{14}C)α-aminoisobutyric acid (AIB) (56.3 mCi mmole^{-1}) were purchased from ICN Biochemicals, Irvine, California. (1-^{14}C)β-alanine (54.7 mCi mmole^{-1}) and L-(U-^{14}C)alanine (174 mCi mmole^{-1}) were obtained from New England Nuclear, Boston, Massachusetts.

Non-radiolabeled amino acids, analogs, ouabain, and N-ethylmaleimide (NEM) were obtained from the Sigma Chemical Company (St. Louis, Missouri), DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, was obtained from Dupont de Nemours and Company (Wilmington, Delaware).

Protocol. Algal cell suspensions were allowed to incubate under standard experimental conditions for one hour, with gentle agitation on a Model 3520 Orbit Shaker (Lab-Line, Melrose Park, Illinois), prior to the addition of radiolabeled compounds.

In all assays, ^{14}C-compounds were added to cell suspensions to obtain a final concentration of 1 μCi ml^{-1}. Immediately after the addition of labeled compounds, aliquots (0.1 ml) were removed to determine the initial level of radioactivity. Triplicate samples (1.0 ml) were subsequently removed at designated time periods and the zooxanthellae collected on Whatman GF/C filter discs using an Amicon Filtration Manifold. Discs were thoroughly washed, allowed to air dry, and placed in scintillation vials to which 10 ml of Aquasol (New England Nuclear) were added. Uptake values were expressed as DPM · μg chl^{-1}ml^{-1} and rates of uptake (slope) as uptake per hour. Samples were counted in a Beckman, Model LS7500, programmable liquid scintillation counter.

In experiments designed to monitor the competition of unlabeled amino acids and analogs with the uptake of L-(U-^{14}C)alanine, unlabeled substrates were added in 5000-fold concentrations to that of alanine. Metabolic inhibitors were used at the following concentrations: KCN, 0.01 m*M;* NEM, 0.5 and 5.0 m*M;* ouabain, 0.1 m*M;* NaF, 1.0 m*M;* and DCMU, 0.001 and 0.01 m*M.*

Distribution of incorporated alanine

To investigate algal utilization of alanine, zooxanthellae were incubated over three hour time periods in L-(1-^{14}C)alanine, washed free of exogenous isotope, and sequentially extracted in 50% ethanol followed by chloroform:methanol (3:1). Triplicate samples (0.4 ml), taken to dryness, were counted immediately after uniform algal suspension in each extractant and the dried supernatants counted to insure the completeness of the operation. Cell densities were maintained at uniform levels by appropriate dilution with each extraction solvent. Radioactivity remaining in the final pellet was determined by difference.

Statistics

Three-hour comparisons were used in this study, since standard linear regression techniques showed that uptake rates for alanine were linear over the course of six hours. Student's *t*-tests and regression analyses were carried out according to Snedecor and Cochran (1971).

RESULTS

Rate of alanine uptake by zooxanthellae

Alanine uptake rates (DPM · μg chl^{-1}ml^{-1}h^{-1}) were determined by measuring the slopes for seven different algal cell suspensions during six hour incubation periods in L-(U-^{14}C)alanine. Uptake measurements were made after the first thirty minutes and after each hour.

Slopes for these experiments were found to be highly linear as indicated by the correlation coefficients (mean ± S.D. = 0.9836 ± 0.0247). A high degree of variability among the rates was noted, however (mean ± S.D. = 1171 ± 914).

Specificity of alanine uptake

Isolated zooxanthellae did not appear to exhibit stereospecificity of alanine uptake (Fig. 1). Comparisons of uptake of D-(1-^{14}C)alanine to that of L-(1-^{14}C)alanine ranged from 101–109% (n = 3), and the 3-hour mean values were not statistically different ($P > 0.10$). These results differ from those reported for various animal cells or purified plasma membrane vesicles where varying, but significant, differences were noted with different amino acids (Sigrist-Nelson *et al.*, 1975; Sacktor, 1977; Im and Spector, 1980; Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981).

The uptake rate for L-(1-^{14}C)alanine compared to the (1-^{14}C)analogs β-alanine and α-aminoisobutyric acid (AIB) is presented for a typical experiment in Figure 2. Both analogs showed highly reduced 3-hour uptake values compared to controls, ranging from 24–31% (n = 2) for β-alanine and 4–24% (n = 4) for AIB.

Ion-dependency of alanine uptake

Uptake of exogenous ^{14}C-alanine was found to be highly dependent on the presence of sodium in the medium. Uptake values after 3-hour incubation periods in sodium-free medium were 3.48 ± 2.57% of controls (n = 4). Thus, approximately 97% of alanine uptake by isolated zooxanthellae was sodium-dependent, which is consistent

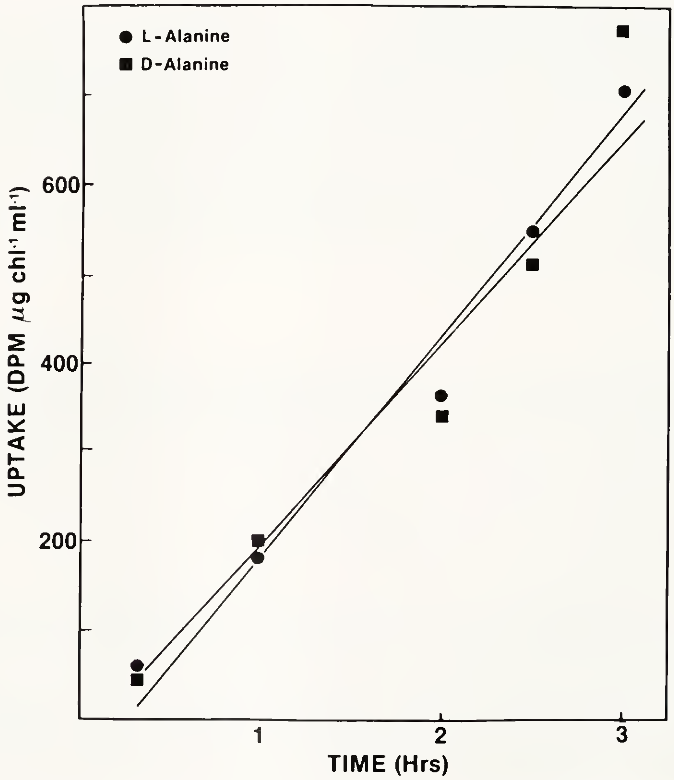

FIGURE 1. Uptake of L- and D-(1-^{14}C)alanine by isolated zooxanthellae.

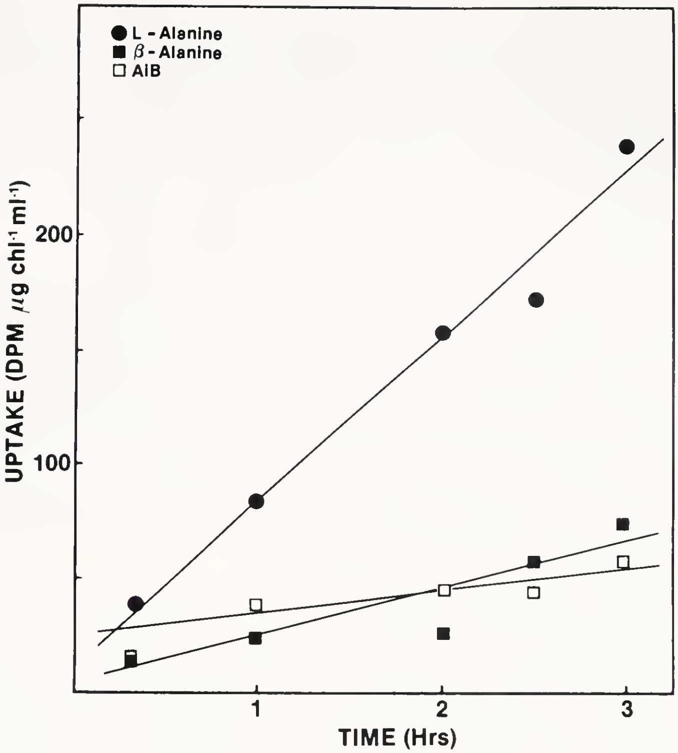

FIGURE 2. Uptake of L-(1-^{14}C)alanine, β-alanine, and α-aminoisobutyric acid (AIB) by isolated zooxanthellae.

with the results reported for this amino acid in a wide variety of cells (Guidotti *et al.*, 1978; Christensen, 1979, 1982).

Uptake systems for alanine in zooxanthellae

Sodium-dependent alanine uptake occurs in a variety of cell types via two systems designated A and ASC by Christensen (1979) and co-workers (Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981). System A functions primarily in the uptake of neutral amino acids with short, polar, or linear side-chains. A sub-set of System A, the ASC System, has the greatest affinity for three such amino acids: alanine, serine, and cysteine. Transport of an amino acid such as alanine by each of these systems can be distinguished by competition experiments using the methylated analog α-methylaminoisobutyric acid (MeAIB). This analog competes for uptake sites of System A but not of System ASC. In contrast to these systems, System L is sodium-independent and transports neutral aromatic amino acids or those that have branching side-chains.

Since these systems are characteristic of a variety of cell types, similar mechanisms in zooxanthellae may provide a basis for the interpretation of alanine uptake in the presence of the competing amino acids or analogs (Table I). The lack of a large reduction of alanine uptake in the presence of MeAIB (10% of control) would argue that the ASC System serves as the major uptake system for alanine in zooxanthellae. The predominance of the ASC System is also consistent with the reduction of alanine

TABLE I

Uptake of L-(U-^{14}C) alanine (174 mCi mmole^{-1}) by isolated zooxanthellae in the presence of 5000-fold molar excess of unlabeled amino acids and analogs

Compound	Mean percent ± S.D.
Control	100.0
L-alanine (4)	48.9 ± 4.2
MeAIB (3)	89.5 ± 24.7
L-serine (4)	65.5 ± 6.8
L-cysteine (3)	73.9 ± 15.3
β-alanine (3)	71.1 ± 1.7
L-valine (3)	88.0 ± 4.0
L-leucine (3)	92.2 ± 7.1

Values represent 3-hour uptake levels (DPM · μg chl^{-1} ml^{-1}), expressed as mean percentage ± S.D. of control levels. Number of experiments are given in parentheses. MeAIB = α-methylaminoisobutyric acid.

uptake in the presence of the amino acids which share this system, L-serine and L-cysteine. In contrast, amino acids transported by System L, such as L-valine and L-leucine, were not as effective as competitors.

Effects of inhibitors on alanine uptake

The effects of inhibitors on the uptake of alanine by isolated zooxanthellae is shown in Table II. Uptake of L(U-^{14}C)alanine was markedly depressed by KCN (0.01) and by NEM (0.5 and 5.0 m*M*), although a significant amount of alanine uptake still occurred in their presence. NEM has been used to inhibit ATPase activity in plants (Leonard, 1982), and ^{3}H-NEM has been used to analyze the role of sulfhydryl group integrity in the Na$^+$,K$^+$-ATPase of mammalian cells (Winslow, 1981). In contrast, the glycolytic inhibitor NaF had no apparent effect. The lack of reduction of alanine uptake in the presence of ouabain, a cardiac glycoside which is extremely effective in inhibiting animal ATPase enzymes, is noteworthy and may reflect differences between the ATPases of plants and animals. In this regard, ATPase insensitivity to ouabain has been reported in several algae and higher plants (Leonard and Hodges, 1973; Sullivan and Volcani, 1974; Hellebust, 1978; Leonard, 1982).

TABLE II

Effects of inhibitors on uptake of L-(U-^{14}C) alanine (144 mCi mmole^{-1}) by isolated zooxanthellae

Inhibitor	Inhibitor concentration (m*M*)	Percentage of control uptake (average)
None	—	100
KCN	0.01	45 (3)
N-ethylmaleimide	5.0	35 (2)
	0.5	27 (2)
NaF	1.0	95 (2)
Ouabain	0.1	110 (2)
DCMU	0.01	97 (4)
	0.001	111 (4)

3-hour uptake values = DPM μg chl^{-1} ml^{-1}. Number of experiments are given in parentheses. DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

The effect of the photosynthetic inhibitor DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, was tested to evaluate the role of photosynthetic production of amino acids on exogenous alanine uptake (Table II). *Cassiopea* zooxanthellae incubated in the presence of ^{14}C-bicarbonate and 0.01 mM DCMU for three hours demonstrated that less than 1% of the isotope was fixed. ^{14}C-alanine uptake, in the presence of this inhibitor, on the other hand, was not significantly reduced ($P > 0.10$), despite the almost complete shutdown of photosynthesis.

Distribution of incorporated alanine

The distribution of alanine in zooxanthellae exposed to L-(1-^{14}C) alanine for three hours shows that the algae are able to metabolize this amino acid into several types of compounds (Table III). Over half the radiolabeled material remained in the insoluble pellet after sequential extraction of the algal cells with ethanol and chloroform:methanol, indicating incorporation into high molecular weight compounds and structural components. Soluble compounds differentially extracted by ethanol and chloroform:methanol would be expected to contain low molecular weight organic and lipid components, respectively.

DISCUSSION

While the translocation of organic nutrients from endozoic algae to their animal hosts is well documented, less attention has been paid to the extent of back-transfer of such molecules. Several studies (Cook, 1971; Thorington and Margulis, 1981) have provided evidence that endozoic algae may compete for the pool of soluble organic nutrients available from host metabolism, especially during periods of photosynthetic inactivity. Previous studies have not characterized the mechanisms involved in the transport of nutrients, such as sugars and amino acids, by zooxanthellae, so that they might be compared to similar systems in non-symbiotic unicellular algae or in animal cells. Studies such as these are essential to our understanding of the maintenance and regulation of these symbiotic associations.

The results of this investigation support the view that isolated zooxanthellae take up alanine by mechanisms similar to those of animal cells, and no systems unique to the symbiotic state appear to be operative. Incorporation studies (Table III) suggest that algal endosymbionts can utilize alanine, and possibly other nutrients present in animal cells pools, for growth and maintenance.

As demonstrated for a wide variety of cell types, uptake by zooxanthellae is highly

TABLE III

Distribution of ^{14}C in isolated zooxanthellae following 3-hour incubation periods in L-(1-^{14}C) alanine

		Solvent	
Experiment	Ethanol	Chloroform:methanol	Algal pellet
1	25.7	26.6	47.4
2	34.8	22.1	43.1
3	20.5	14.1	65.4
4	20.4	16.3	59.7
Mean ± S.D.	25.4 ± 6.7	19.8 ± 5.7	54.0 ± 10.4

Values represent percentage of total radioactivity extracted by the solvent or remaining in the final wash pellet, for 4 separate experiments. Totals are mean percent ± S.D.

Na^+-dependent. Although stereospecificity appears to be lacking, reduced uptake rates of alanine analogs demonstrate that there is transport specificity. This is further supported by competition experiments using several natural amino acids and analogs (Table II) which exhibited varying degrees of competitive inhibition. Such experiments may also be interpreted in terms of the systems of amino acid transport described in studies by numerous investigators on a variety of cells (Christensen, 1979; Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981; Shotwell *et al.*, 1981; Christensen, 1982). System ASC appears to be the primary transport mechanism for alanine since MeAIB, an inhibitor of System A but not ASC, was ineffective in suppressing uptake. This conclusion is also supported by the observations that serine and cysteine, which, along with alanine, are most effectively transported by System ASC, were the most effective competitors. Amino acids which are transported by the Na^+-independent System L, such as valine and leucine, did not significantly reduce uptake.

It should be noted that the ratio of these uptake systems varies widely among the many types of cells studied and may not remain constant within a given cell type. Adaptive changes in the activity of System A in animal cells have been demonstrated with respect to varying exogenous levels of relevant amino acids (Gazzola *et al.*, 1981; Shotwell *et al.*, 1981). Cells incubated under conditions of amino acid deprivation demonstrated increased System A activity, while those exposed to high levels of exogenous amino acids showed depressed uptake. These responses were not found for System ASC. Such adaptive responses, if present in zooxanthellae, could account for the variability of uptake rates seen in different algal cell suspensions. Variability may also be due to differences in *in situ* preconditioning by animal host factors as reported in the following paper (Carroll and Blanquet, 1984).

The question of whether uptake of exogenous alanine may be affected by the endogenous production of amino acids through photosynthesis was addressed in experiments utilizing the photosynthetic inhibitor DCMU. The results of these assays indicated that uptake of ^{14}C-alanine in the presence of DCMU was not significantly different from controls. In analogous investigations using animal cells, the uptake of amino acids was unaffected after preloading cells with these compounds in order to elevate artificially internal levels (Guidotti *et al.*, 1978).

Some features of alanine uptake by zooxanthellae, exemplified by the effects of inhibitors, were found to differ from animal cells. In their review, Kimmich and Carter-Su (1978) proposed a model in which differences in both the ionic and electrical potentials across plasma membranes play important roles in energizing the concentrative uptake of sugars and amino acids. While electrogenic potentials are generated by membrane ATPase activity, it should be noted that the properties of this enzyme differ in plants and animals. In general, higher plants and algae possess a K^+-ATPase, where the K^+ influx does not appear to be coupled to Na^+ efflux, as occurs in animal cells (Leonard, 1982). Other studies suggest that electrogenic extrusion of H^+ is a prominent feature of plant cells (Poole, 1978; Smith and Raven, 1979), and it is possible that the efflux of H^+, instead of Na^+, is coupled to K^+ transport. If the ATPase is purely an electrogenic proton pump, an antiport mechanism could facilitate an uphill Na^+ efflux. Such a mechanism has been identified in *Halobacterium halobium*, where a symport for Na^+-alanine uptake is coupled to an antiport for H^+ and Na^+ (Lanyi and MacDonald, 1976). Such a coupled mechanism could account for the ouabain-resistant, sodium-dependent uptake of alanine observed in zooxanthellae.

The effect on alanine uptake in zooxanthellae by exogenous KCN is also noteworthy (Table II). While KCN markedly depressed alanine uptake, a significant uptake occurred in its presence. A possible explanation is the continued production of ATP via a cyanide-resistant respiration pathway, which has been reported in some algae, higher plants and animals (Lloyd, 1974; Henry and Nyns, 1975; Zaba, 1983). Such respiration occurs by an alternative electron transport route, which arises after the first oxidative phosphorylation site, and allows the transport of electrons to oxygen without further ATP production. The existence of such a pathway, however, has recently been questioned (Kelly, 1982).

There is increasing evidence suggesting that endozoic algae compete for and utilize nutrients available in animal cell pools. However, the extent to which the stability of symbiotic associations depends upon the regulation of such reciprocal transfers is not known. Investigations focusing on such questions are necessary for a fuller understanding of such symbioses.

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ALANINE UPTAKE BY ISOLATED ZOOXANTHELLAE OF THE MANGROVE JELLYFISH, *CASSIOPEA XAMACHANA*. II. INHIBITION BY HOST HOMOGENATE FRACTION

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ABSTRACT

A low molecular weight fraction (Inhibitory Fraction, IF) was isolated from whole animal homogenates of the symbiotic jellyfish, *Cassiopea xamachana.* This fraction (2–10 kDa) strongly inhibited the uptake of L-(U-^{14}C)alanine by isolated zooxanthellae at concentrations as low as 5 μg protein ml^{-1}. The magnitude of suppression was unaffected by the length of exposure time to IF prior to monitoring uptake. Uptake rates were linear for control and experimental cell suspensions, suggesting that algal metabolic integrity was maintained in the presence of IF. Zooxanthellae incubated in IF, and then washed free of this fraction, continued to show suppressed alanine uptake with only partial recovery. These cells, when placed in culture media, exhibited normal morphology, continued to grow and divide, and developed flagellated stages. The possible regulation of nutrient exchange between symbionts by symbiosis-specific host factors is discussed.

INTRODUCTION

Algal-invertebrate associations provide unique opportunities for investigating the regulation of the metabolic interactions necessary for the maintenance of this symbiosis in two such diverse organisms. While it has been recognized that endozoic algae translocate photosynthate to their animal hosts (for reviews, see Muscatine, 1974, 1980), it has only recently been appreciated that they also may compete for the available pool of nutrients obtained via host feeding (Cook, 1971; Thorington and Margulis, 1981). Since the activity of various metabolic pathways is directed by the availability of substrate, the stability and widespread occurrence of algal-invertebrate symbioses may result from an ability of the symbionts to regulate reciprocal transfers. Few studies, however, have focused on this regulation and the mechanisms involved.

An intriguing possibility is that this might be accomplished, in part, through the induction of certain regulatory molecules or factors specific to the symbiotic state. Factors that increase the rate of photosynthate liberation by isolated zooxanthellae have been reported in various cnidarians (Trench, 1971; Muscatine *et al.,* 1972). In addition, these factors appear to lack species specificity (Muscatine, 1967). The present investigation shows that a homogenate fraction of the jellyfish *Cassiopea xamachana* can significantly inhibit the back-transfer of nutrients such as alanine to zooxanthellae. Should such factors be common to algal-cnidarian associations, their differential production could conceivably regulate the levels of organic compounds available to each partner. The regulation of nutrient availability to zooxanthellae, or the production of appropriate host factors, could also serve to regulate algal populations within cells.

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Steele and Goreau (1977) have reported that a protein obtained from a distilled water extract of the anemone *Phyllactis* reduced the concentration of zooxanthellae suspensions from this species, as well as *Aiptasia* and *Zoanthus,* although *Aiptasia* lacked this protein.

MATERIALS AND METHODS

Cassiopea xamachana were obtained from commercial sources and maintained in the laboratory as previously described (Carroll and Blanquet, 1984). Whole animal homogenates, prepared via mechanical disruption in an Osterizer blender, were subsequently centrifuged to remove the zooxanthellae and other particulate material. A low molecular weight fraction of the resulting supernatant was obtained by vacuum filtration using Millipore immersible CX-10 Ultra-filtration units having a molecular weight cut-off of 10 kDa. Extensive dialysis of the filtrate in benzoylated tubing (Sigma, St. Louis, Missouri) against filtered sea water (FSW) removed all compounds less than 2 kDa. This fraction (2–10 kDa), referred to as Inhibitory Fraction (IF), significantly suppressed the uptake of exogenous alanine by zooxanthellae. Concentration of IF was determined by protein measurement according to the methods of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Effect of IF on alanine uptake by zooxanthellae

Cassiopea zooxanthellae were freshly isolated for all experiments according to the methods of Blanquet *et al.* (1979). Cell suspensions were adjusted to standard densities of $3 \cdot 10^6$ cells ml^{-1}. Total chlorophyll (chl a and c_2) was determined by the methods of Jeffrey and Haxo (1968). Cell suspensions, in the presence or absence of IF, were allowed to preincubate for one hour with gentle agitation under standard conditions of light (45 ± 3 μEinsteins m^{-2} s^{-1}) and temperature (23 ± 2°C) prior to the addition of L-(U-^{14}C) alanine (144 mCi $mmole^{-1}$) to obtain a final concentration of 1 μCi ml^{-1}. Radiolabeled alanine was obtained from ICN Biochemicals (Irvine, California).

The effects of IF (1 to 100 μg protein ml^{-1}) on the uptake of alanine were monitored after 3-hour incubation periods. In all experiments, triplicate samples were prepared as previously reported (Carroll and Blanquet, 1984) and counted in a Beckman (Model LS7500) programmable liquid scintillation counter.

To determine whether the length of time of exposure of isolated zooxanthellae to IF affected subsequent uptake of L-(U-^{14}C) alanine, standard algal suspensions were divided into two fractions. Fraction I (control) contained algae suspended in FSW, while algae in fraction II were exposed to IF (50 μg protein ml^{-1}) for periods of time ranging from 9 to 180 minutes. The latter were subsequently washed free of the inhibitor prior to the addition of alanine to both fractions. In these experiments the one-hour preincubation step was omitted.

Effects of inhibitory fraction previously used in inhibition of alanine uptake

Assays were performed to determine whether IF retained maximal activity throughout the course of uptake experiments. In these assays, freshly isolated zooxanthellae were divided into two fractions, each of which were incubated under standard experimental conditions for one hour. Fraction I (control) contained standard concentrations of suspended algae in sea water without IF, while Fraction II contained sea water with IF (50 μg protein ml^{-1}). At time zero, aliquots (20 ml) of each fraction were put into separate beakers and L-(U-^{14}C)alanine (1 μCi ml^{-1}) was added to each.

Uptake was monitored at intervals throughout the experiment. At 2.3 hours, ^{14}C-alanine was added to an additional aliquot (20 ml) taken from Fraction I, and uptake again monitored. In addition, zooxanthellae from Fraction I were washed in fresh sea water and resuspended to standard densities either in a supernatant from Fraction II, termed "used IF," or in IF not previously exposed to zooxanthellae termed "fresh IF." Radiolabeled alanine was added and uptake again monitored.

Recovery of alanine uptake after prior exposure to inhibitory fraction

The reversibility of IF inhibition of alanine uptake in zooxanthellae was experimentally determined by dividing cell suspensions into two fractions. Fraction I (control) contained no IF, while Fraction II contained IF at a concentration of 50 μg protein ml^{-1}. L-(U-^{14}C) alanine was added at time zero. After 2 hours, two measured aliquots were removed from Fraction II. One of the aliquots was washed three times in FSW containing the experimental concentration of IF; the other aliquot was similarly washed, using FSW to remove the IF previously present. L-(U-^{14}C) alanine was added to these aliquots at the initial experimental concentration, and uptake was again monitored.

RESULTS

Low molecular weight fractions (2–10 kDa), prepared from *Cassiopea* homogenates, were found to suppress significantly the uptake of exogenous alanine by isolated zooxanthellae at concentrations as low as 5 μg protein ml^{-1} (Fig. 1). Concentrations above 25 μg protein ml^{-1} showed near maximum effectiveness, and 50 μg protein ml^{-1} was chosen as the standard concentration for subsequent experiments. The magnitude of uptake suppression appeared to be unaffected by the length of exposure time (9–180 minutes) to this fraction (Table I). Comparison of 3-hour uptake values of paired samples of pre-dialysis (0–10 kDa) and post-dialysis (2–10 kDa) fractions

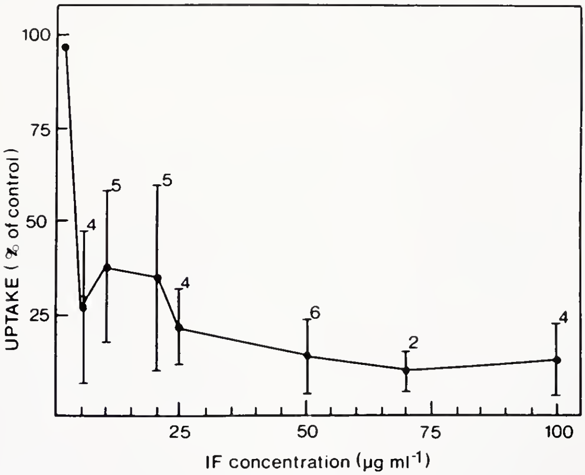

FIGURE 1. Uptake of L-(U-^{14}C) alanine by isolated zooxanthellae in the presence of different concentrations of Inhibitory Fraction (IF). Data points are mean values ± S.D. as percentage of control. Data point at 1 μg protein ml^{-1} is the mean of two experiments. The number of experiments for other concentrations are given above the bars.

TABLE I

Effect of length of exposure to Inhibitory Fraction (IF) on subsequent uptake of L-(U-^{14}C) alanine by isolated zooxanthellae

Experiment	Control slope	Experimental slope		
1	461	205 (9)	214 (25)	168 (60)
2	511	264 (10)	284 (80)	293 (180)
3	802		397 (60)	405 (120)
4	428		126 (60)	108 (80)
5	503		143 (25)	134 (40)

Uptake rates (slope) are expressed as DPM · μg chl^{-1} ml^{-1} h^{-1}. Exposure times (in minutes) are given in parentheses.

of individual homogenates (Table II) showed no significant difference ($P > 0.10$), indicating that there are no active molecules smaller than 2 kDa.

As shown here and in previous experiments (Carroll and Blanquet, 1984), the rate of alanine uptake by isolated zooxanthellae was highly linear. This was also true for algae either continuously exposed to IF or after previous exposure to this fraction (Figs. 2, 3). Correlation coefficients for the slopes of all experiments were greater than 0.97. As shown in Figure 2, IF retained its effect over the course of a 3-hour incubation with zooxanthellae, and could subsequently inhibit fresh algal suspensions to the same extent.

Zooxanthellae which were washed free of IF after a 2-hour exposure period continued to demonstrate suppressed uptake, with partial recovery, when resuspended in FSW at initial alanine concentrations (Fig. 3). Algal cells washed in IF and resuspended in IF, showed uptake rates similar to those prior to washing.

Viability of zooxanthellae exposed to IF for 2–4 hours was monitored by innoculating washed cells into FSW containing Carolina Biological Alga-Gro Concentrate (20 ml liter^{-1}). Aliquots from all cultures, examined by phase contrast microscopy after 2–5 days, contained cells of normal morphology and size, actively dividing stages, and numerous flagellated forms. In addition, the linear uptake rates noted in all experiments suggest that the metabolic integrity of the zooxanthellae remained intact during exposure to IF.

TABLE II

Inhibition of uptake of L-(U-^{14}C) alanine by Pre- and Post-dialysis Inhibitory Fraction

Experiment	Control	Pre-dialysis IF	Post-dialysis IF
1	1162.7	516.3 (44.4%)	575.8 (49.5%)
2		376.5 (32.4%)	423.7 (36.4%)
3	2009.2	344.7 (18.2%)	470.1 (23.4%)
4		351.5 (17.5%)	347.6 (17.3%)
Mean ± S.D.		397.3 ± 80.5	454.3 ± 95.5

Uptake values are expressed as DPM · μg chl^{-1} ml^{-1} for 3-hour samples. Percentage of control is given in parentheses.

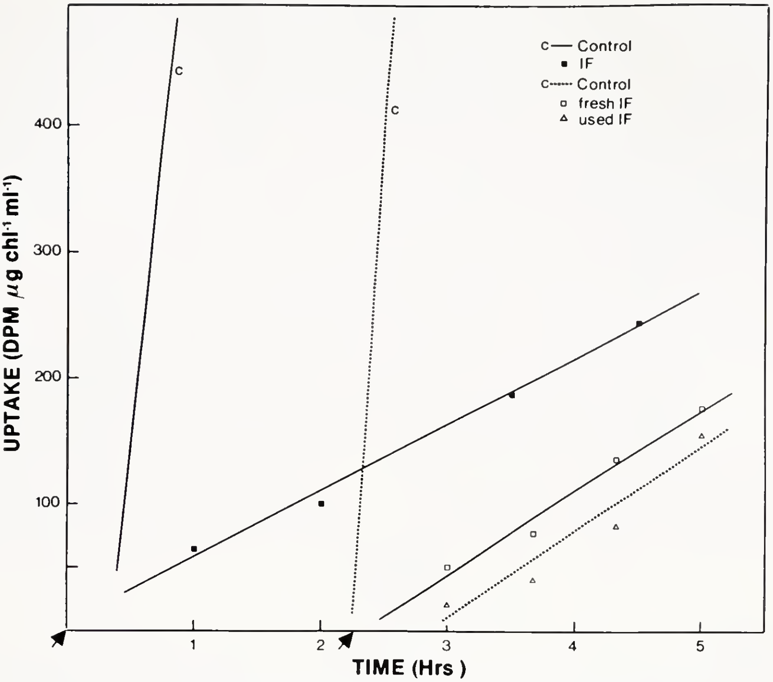

FIGURE 2. Isolated zooxanthellae were divided into two fractions, one of which contained no added IF (Fraction I), the other (Fraction II) contained IF at a concentration of 50 µg protein ml^{-1}. At time zero (first arrow), ^{14}C-alanine was added to 20 ml aliquots from Fraction I (C —— and Fraction II (■ IF) and uptake monitored. Again at 2.3 hours (second arrow) ^{14}C-alanine was added to an additional aliquot from fraction I (C – – –) and uptake rate determined. In addition, algae from fraction I were washed in FSW and resuspended either in a supernatant from Fraction II, termed "used IF" (△), or in IF not previously exposed to zooxanthellae, "fresh IF" (□), to which radiolabeled alanine was added. Each regression line was drawn to the calculated slope obtained from 5 data points, although not all points are shown on each line. Regression analyses were carried out according to Snedecor and Cochran (1971).

DISCUSSION

The results of this study indicate that a fraction from *Cassiopea xamachana* homogenates (IF) is able to suppress significantly the uptake of alanine by zooxanthellae *in vitro* and thus provide a means by which back-transfer of nutrients *in vivo* may be controlled. While IF inhibits amino acid uptake, it has little or no effect on photosynthesis (Blanquet, unpub.), indicating that IF is not acting as a general metabolic inhibitor. Since aposymbiotic *Cassiopea* were not available for this investigation, it was not possible to determine whether production of IF is symbiosis-specific, as has been shown for host factors in other cnidarians (Trench, 1971).

The possibility that the measured suppression of ^{14}C-alanine uptake in the presence of IF was due to a reduction in the specific activity of exogenous, radiolabeled alanine through the enhanced release of this amino acid from zooxanthellae by some component of IF does not appear to be tenable. Trench (1974) and Muscatine and Cernichiari (1969) have shown that alanine is a minor component of the photosynthate released from zooxanthellae in the presence of cnidarian host homogenates. The latter investigators demonstrated that, in the coral *Agaricia,* only 7.2% of the fixed ^{14}C released in one hour by host homogenate was in the form of alanine. Though this

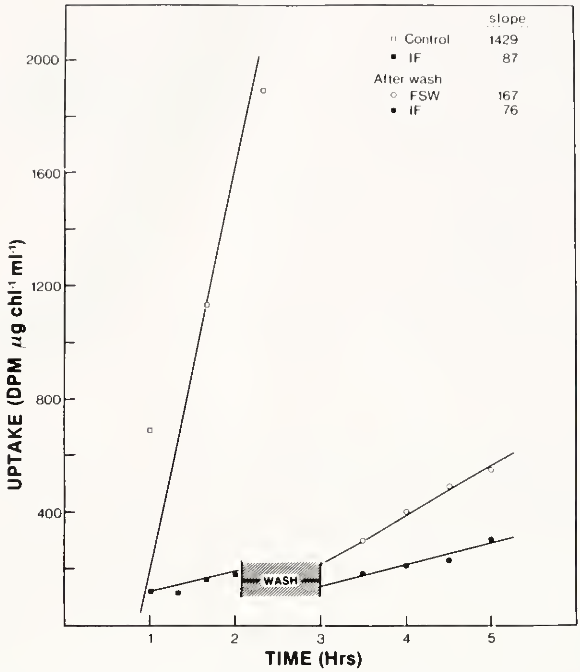

FIGURE 3. Uptake of L-(U-^{14}C) alanine by isolated zooxanthellae in medium without IF (□) or with IF (■). At 2 hours, cells were washed, either in the experimental concentration of IF (●) or in FSW (O). Cells were then resuspended in the appropriate medium to which ^{14}C-alanine was added and uptake again monitored.

percentage increased with time, the apparently small amounts of alanine released from these algae, together with the magnitude of suppression and linearity of alanine uptake by *Cassiopea* over three hours in the presence of IF, would argue against the above interpretation. In addition, alanine uptake remains suppressed after IF has been removed (Fig. 3).

Inhibitory fraction was Lowry-positive, with a molecular weight range of 2–10 kDa, and thus it is probable that the active factor(s) may be peptides or low molecular weight proteins. This fraction was shown to be effective at concentrations as low as 5 μg protein ml^{-1}. Since it is reasonable to assume that IF consists of several components, not all of which may be active in amino acid uptake regulation, the active component(s) would be present at even lower concentrations. Therefore, to inhibit effectively back-transfer of amino acids, animal cells *in vivo* may need to synthesize only small amounts of the inhibitory component(s) of IF. Alternatively, host cells might produce and sequester greater amounts of IF, which could then be released as needed to regulate amino acid levels. Such a strategy, which is characteristic for various neurosecretory peptides and endocrines, would enable the host cells to have a faster response time than if *de novo* synthesis of the inhibitor were required. On the other hand, decreased production or release of IF would allow increased uptake during periods of excess amino acid availability, thereby enhancing the recycling of nutrients.

Internalization or down-regulation of putative IF-alanine receptor complexes by zooxanthellae could also supply a means for IF degradation, thereby allowing a return to a baseline condition from which subsequent adjustments could be made. The persistence of suppressed alanine uptake after the removal of IF (Fig. 3) would support this hypothesis. Indeed, internalization and degradation of numerous polypeptides through coated pits has been well documented in animal cells (Schlessinger *et al.*, 1978; Branca *et al.*, 1982; Krupp *et al.*, 1982; Blanchard *et al.*, 1983).

In this regard, the observed variability in control slopes and in IF suppression (Table I) appears to reside mainly in the differential response of individual cell suspensions due to *in situ* preconditioning in host cells. The possibility that variability resides solely in concentration differences of active components within specific IF preparations is negated by observations that the same IF preparation used with different suspensions of isolated zooxanthellae demonstrated variable suppression.

Regulation of algal activity by animal host factors would require some degree of symbiont specificity. Thus, it would be unlikely that the IF of *Cassiopea* would act as a general metabolic inhibitor, since host cells and organelles would also be affected by its presence. It is more likely that IF either specifically modulates the action of algal alanine uptake systems or, in some way, inhibits algal ATPase activity. The differences observed between ATPases of plants and animals (Leonard, 1982) may provide a basis for such selective inhibition. Studies currently underway on the chemical nature and properties of IF will help to elucidate its mechanism of action.

ACKNOWLEDGMENTS

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THE EFFECTS OF TEMPERATURE AND WATER AVAILABILITY ON ION AND ACID-BASE BALANCE IN HEMOLYMPH OF THE LAND HERMIT CRAB *COENOBITA CLYPEATUS*

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ABSTRACT

Temperature acclimation (18–30°C) and dehydration (to 86% of initial mass) are two problems frequently encountered by the land hermit crab, *Coenobita clypeatus*. Their individual and combined effects on hemolymph ion and acid-base status were assessed. With free access to 10% SW, crabs maintained a constant degree of hydration, with hemolymph marginally hypo-osmotic to full strength SW. Increased acclimation temperature produced a reduction in pH, characteristic of ectotherms and consistent with maintenance of relative alkalinity which was accomplished by an elevation of CO_2 tension (P,CO_2). Hyperactivity resulted in some spillage of shell water and affected Cl^- balance. Under water deprivation, evaporative loss declined approximately exponentially and was negatively correlated with body mass. Hemolymph osmolality and electrolyte levels were significantly increased, ionic imbalance contributing largely to the hemolymph acidosis. Hemoconcentration was less marked when combined with temperature acclimation. Temperature-dependent pH regulation however in dehydrated crabs was accomplished as in hydrated crabs by ventilatory P,CO_2 control typical of air-breathers. The aquatic route of acid-base regulation (by ionic exchange) potentially afforded by the reservoir of water held in the molluscan shell was apparently not utilized.

INTRODUCTION

Adaptations allowing the transition of crustaceans onto land have evoked considerable study in an attempt to pinpoint criteria for terrestrial evolution. Morphological (Harms, 1932; Gray, 1957) and ecological surveys (Pearse, 1929; Gordon, 1956) have been supplemented more recently with investigations of physiological processes such as hydromineral regulation (Gross, 1963; de Wilde, 1973), nitrogen excretion (Gifford, 1968), respiratory gas exchange (Cameron and Mecklenburg, 1973; Cameron, 1975), and acid-base balance (McMahon and Burggren, 1981). Early advances were summarized by Bliss and Mantel (1968) while the most current information is the comprehensive physiological report of the Alpha Helix expedition to study the land crabs of the Palau Islands (see Cameron, 1981).

The two greatest problems facing terrestrial poikilotherms are no doubt temperature variation and dehydration (Edney, 1960). These two environmental features frequently

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Abbreviations: P,CO_2, partial pressure of CO_2; C,CO_2, total CO_2 content; pK'_1, apparent first dissociation constant of carbonic acid; α,CO_2, solubility coefficient of CO_2; $\Delta H^+m/c$, quantity of H^+ added by metabolic/respiratory acids; β, non-bicarbonate buffering capacity; ECFV, extracellular fluid volume; SID, strong ion difference.

interact in the natural terrestrial environment, yet this interdependence has rarely been considered in the study of physiological compensations. It was the purpose of the present study to examine the aspect of their combined action in the land hermit crab *Coenobita clypeatus,* which may owe its success in the terrestrial environment to the retention of the molluscan shell. This is generally water-filled and may confer resistance against desiccation (Reese, 1969; McMahon and Burggren, 1979).

Amongst the terrestrial decapods, studies have focused primarily on the Brachyura, especially *Cardisoma* and *Gecarcinus,* which are conspicuous in the supralittoral zone of the tropics and subtropics, and also on the monospecific anomuran genus *Birgus.* The terrestrial hermit crabs have received relatively little attention, perhaps because the adopted molluscan shell hinders physiological investigation. McMahon and Burggren (1979) studied respiratory gas exchange in *Coenobita* and investigated the individual effects of temperature acclimation (McMahon and Burggren, 1981) and dehydration (Burggren and McMahon, 1981) on acid-base balance.

Blood pH in ectothermic animals varies inversely with temperature and is actively regulated, according to the alphastat theory advanced by Reeves (1972, 1977). Water- and air-breathers, however, differ with respect to the mechanism of this regulation. Air-breathing vertebrates are thought to alter pH by adjusting the lung ventilation/CO_2 output ratio (*i.e.,* P,CO_2) on the principle of a closed, constant CO_2 system (Howell *et al.,* 1970; Jackson, 1971). Ventilatory control of blood P,CO_2 is not feasible in water-breathers since, in order to satisfy the O_2 requirement, ventilation is comparatively large and blood P,CO_2 is held correspondingly low since water-breathers hyperventilate with respect to CO_2 excretion requirements (Rahn, 1966). Instead, an open system usually operates to control the level of bicarbonate (McMahon *et al.,* 1978).

Since the bimodal breather *Coenobita* has access to both air and water from stores within the adopted shell, it could utilize either aerial (*i.e.,* control of P,CO_2) or aquatic (control of HCO_3^-) mechanisms of acid-base regulation. In an earlier study, McMahon and Burggren (1981) suggested that both mechanisms could operate in controlling extracellular pH. The purpose of the present study was to investigate whether the relative overall contribution of the two mechanisms reflects water availability.

MATERIALS AND METHODS

Animal maintenance

The investigation was conducted on 26 individuals of *Coenobita clypeatus* (Herbst), identified using Bright (1966). For 2 weeks prior to experimentation they were housed in a 1.3 × 0.4 × 0.6 m terrarium maintained at 30 ± 1°C (R.H. near to 40%). Crabs were fed liberally until the commencement of the experiment and provided with 10% sea water, the salinity they prefer (de Wilde, 1973).

Experimental design

The extremes of temperature studied were 18° and 30°C, which encompass the natural distribution of this species (Provenzano, 1959; de Wilde, 1973). Since ambient temperature varied, crabs were dehydrated to a constant water loss ($\simeq$14% of original body mass) sufficient to significantly elevate hemolymph osmolality (Burggren and McMahon, 1981). Total body mass (including shell water) was estimated as the difference between total mass (*i.e.,* shell plus animal) and the mass of the shell, since enforced shell evacuation is feared to affect the animals adversely. The mass of the inhabited *Livona* shells could be predicted confidently from external morphometric measurements according to the procedure outlined by Wheatly (1984). Rehydration was subsequently examined. For comparison with the individual effect of temperature acclimation and desiccation, their combined action was investigated. Details of the experimental protocol are provided in Figure 1. Criteria considered in this design were; at least 4 days for temperature acclimation (Truchot, 1978) and rehydration, 2 days recovery from sampling prior to water deprivation, and 4 days between any two consecutive samples to avoid effects of repetitive sampling.

Hemolymph sampling and analytical procedures

Total mass (*i.e.*, animal plus shell) was recorded daily, always prior to sampling. 750–800 μl of prebranchial hemolymph was drawn into a 1 ml Hamilton gastight syringe with a 22-gauge needle inserted through the arthrodial membrane between the meropodite and coxopodite of the larger cheliped which was the only accessible site.

Osmolality and Cl^- concentration were determined immediately using a vapor pressure osmometer (Wescor 5100B) and digital chloridometer (Searle Buchler 4-2500) respectively. 100–200 μl of hemolymph were frozen rapidly and inorganic cations determined later by atomic absorption spectrophotometry (Jarrell-Ash 850). Mg^{2+} and Ca^{2+} were diluted 1 in 3000 and 500 respectively with 0.1% $LaCl_3 \cdot 6H_2O$, and K^+ and Na^+ were diluted 1 in 500 and 8000 respectively in 0.1% CsCl to suppress interferences in the air-acetylene flame.

pH was determined immediately on a 50 μl subsample using a liquid junction capillary electrode (Radiometer, G299A) connected to an acid-base analyzer (Radiometer PHM 71), calibrated against precision buffers and thermostatted to the appropriate temperature. Total CO_2 (C,CO_2) was measured on a 50 μl subsample using a Corning 965 CO_2 analyzer calibrated against standard bicarbonate solutions of 15 and 30 mmol l^{-1}. P,CO_2 was measured directly using a Radiometer electrode (E 5037-

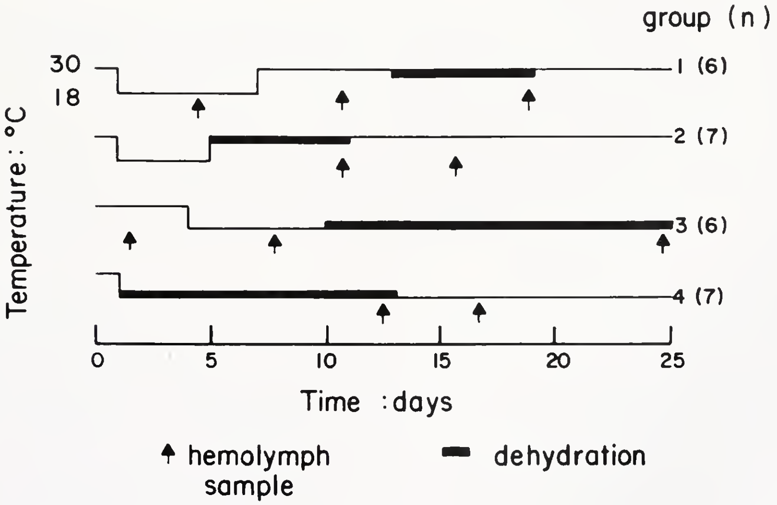

FIGURE 1. Diagrammatic representation of experimental protocol used to determine the responses of *Coenobita clypeatus* to a change in ambient temperature with and without access to free water, and progressive dehydration and rehydration at each acclimation temperature.

0) thermostatted to experimental temperature using a procedure modified from that of deFur *et al.* (1980). To minimize depletion of CO_2 from the sample and P,CO_2 gradients between hemolymph and electrolyte, two consecutive aliquots of $\simeq 100\ \mu l$ were flushed through the chamber at timed intervals, the syringe remaining in position to prevent introduction of air bubbles. At 18°C, intervals of two to three minutes were used while at 30°C, when equilibration was faster, two one minute periods were sufficient. The electrode was calibrated with millipore-filtered crustacean Ringer's solution equilibrated with humidified 1 and 2% CO_2 gas mixtures obtained from Wösthoff mixing pumps and P,CO_2 displayed on an acid-base analyzer (PHM 71) set to increased sensitivity (×2).

For the determination of lactate concentration 80 μl of hemolymph were precipitated in 400 μl of cold 12% perchloric acid, centrifuged, and the supernatant analyzed using a commercial kit (Sigma Technical Bulletin No. 826-UV). Serum protein concentration was determined by the Biuret method (Levin and Brauer, 1951) using a combined albumin/globulin standard (80 mg ml^{-1}).

Derived variables

(i) Molecular $CO_2 = \alpha CO_2 \cdot P,CO_2$—$\alpha CO_2$ is the solubility coefficient for CO_2 taken from Truchot (1976a) at the appropriate temperature and hemolymph ionic strength.

(ii) $[HCO_3^-] = C,CO_2 - \alpha CO_2 \cdot P,CO_2$.

(iii) $OH^-/H^+ = 10^{2(pH-pN)}$—an expression of "relative alkalinity" (see Howell *et al.*, 1973) where pN = 1/2 pKw (Austin and Cullen, 1925).

(iv) Δvariable/Δt—the coefficient of temperature variation.

(v) $\beta = \Delta[HCO_3^-]/\Delta pH$—the non-bicarbonate buffer capacity was calculated from the measured protein concentration using an equation derived for *Carcinus* by Truchot (1976b). McMahon and Burggren (1979) found that this equation was applicable to *Coenobita,* producing values similar to those measured in other land crabs (*e.g.,* Smatresk *et al.,* 1979).

(vi) pK'_1—Difficulties may arise from the use in analysis of apparent first dissociation constants of carbonic acid (pK'_1) determined in other species (see Wilkes *et al.,* 1980; Wheatly and McMahon, 1982). There was a considerable discrepancy between P,CO_2 calculated using pK'_1 for *Carcinus* (Truchot 1976a—see below) and the values presently measured. For this reason, to enable construction of a classical $[HCO_3^-]$ *versus* pH analysis, operational pK'_1 values were calculated for each treatment by substitution into the Henderson-Hasselbalch equation. If pK'_1 did not change significantly as a result of the experimental treatment values were combined for construction of P,CO_2 isopleths.

(vii) ΔH^+c and ΔH^+m—quantities of H^+ added by respiratory (c) and metabolic (m) acids respectively were estimated as in McDonald *et al.* (1979). Wherever the location of P,CO_2 isopleths were altered significantly as a result of the treatment, the point of intersection of the original P,CO_2 at the final location of the buffer line was considered. Paired observations were not available for groups 2 and 4 whose mean values were thus analyzed against the partner group (hence no variability).

(viii) $\Delta[HCO_3^-]$ and $\Delta[Ca^{2+}]$—changes in hemolymph $[HCO_3^-]$ and $[Ca^{2+}]$ in addition to those arising as a spurious consequence of hemoconcentration/dilution. In calculating the latter the assumption was made that changes in hemolymph osmolality were representative of changes in circulating volume.

Statistical treatment of data

Data are given as mean ± SEM (n) and Student's *t*-test performed on paired variates using $P < 0.05$ as the confidence level. Wherever values are presented as a percentile, statistical computations were performed on raw data. Linear regression (least squares method) was performed where appropriate.

RESULTS

Osmo- and ionoregulation

Temperature acclimation in hydrated crabs. Changes in mean body mass resulting from temperature acclimation and hemolymph sampling (groups 1 and 3) are given in Table I and compared with values for rehydrated crabs (groups 2 and 4). On the basis of previous work (see Weymouth *et al.*, 1944; Scholander *et al.*, 1953), errors introduced from weight loss due to metabolic carbon were considered negligible in the present study and changes in mass were assumed to represent body and/or shell water. Individual animals with free access to water maintained a relatively constant mass, with daily variation around the mean of 0.2–1.2 g (equivalent to 0.4–2.0%). Crabs acclimated to 30°C weighed significantly less (−2.5 g). Hemolymph sampling resulted in a loss of 3.6 g body water at 18°C and 1.0 g at 30°C. No overhydration occurred when crabs in groups 2 and 4 were rehydrated.

Corresponding hemolymph osmolality and ion levels are detailed in Table II. There were few significant differences between hydrated groups at the same temperature. $[Cl^-]$ in group 3 was higher at 18°C and lower at 30°C than group 1. $[Ca^{2+}]$ was significantly lower in rehydrated crabs at both 18°C and 30°C and $[Cl^-]$ was higher following rehydration at both temperatures by as much as 70 m-equiv l^{-1} at 18°C. Acclimation to a change in temperature produced a significant increase in

TABLE I

Mean and variation in mass of hydrated Coenobita clypeatus *acclimated to 18 or 30°C both prior to and subsequent to hemolymph sampling*

	18°C		30°C	
Group	Pre	Post	Pre	Post
Group 1 n = 6				
Mass x̄ ± SEM	78.4 ± 5.0	74.7 ± 4.3	72.1 ± 4.0	71.9 ± 4.1
Variation x̄ ± SEM	0.6 ± 0.1	0.3 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Group 2 n = 7				
Mass x̄ ± SEM	75.2 ± 6.5		74.7 ± 7.1*	
Variation x̄ ± SEM	0.5 ± 0.1		0.8 ± 0.2	
Group 3 n = 6				
Mass x̄ ± SEM	59.5 ± 4.4	57.1 ± 5.0	59.2 ± 3.4	57.5 ± 4.5
Variation x̄ ± SEM	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	1.2 ± 0.5
Group 4 n = 7				
Mass x̄ ± SEM	78.9 ± 9.4*		80.4 ± 10.0	
Variation x̄ ± SEM	0.4 ± 0.1		—	

* Rehydrated animals. The units are g throughout.

TABLE II

Hemolymph inorganic ion concentrations and osmolality in hydrated and rehydrated hermit crabs at 18 and 30°C

		18°C							30°C					
		mOsm Kg^{-1}	m-equiv l^{-1}						mOsm Kg^{-1}	m-equiv l^{-1}				
		OSMOLAL	$[Cl^-]$	$[Na^+]$	$[K^+]$	$[Mg^{2+}]$	$[Ca^{2+}]$		OSMOLAL	$[Cl^-]$	$[Na^+]$	$[K^+]$	$[Mg^{2+}]$	$[Ca^{2+}]$
Group 1	x̄	969	337	376	8	59	23		973	387*	375	9	54	28
(n = 6)	± SEM	±15	±11	±7	±1	±1	±2		±22	±13	±10	±1	±3	±1
								→						
2 Rehydrated									993	402**	378	10	49	21**
(n = 7)									±17	±8	±8	±0	±2	±1
3		941	383†	384	8*	60*	26		1005	352†	383	10	52	24
(n = 6)		±23	±6	±10	±1	±3	±1	←	±18	±7	±6	±1	±0	±1
4 Rehydrated		998	411**	367	7	59	19**							
(n = 6)		±21	±10	±18	±0	±5	±1							

The arrow indicates the direction in which the temperature change was effected. (Values are mean ± SEM.)

* Significant differences (paired) arising from a change in acclimation temperature of hydrated crabs.

** Significant differences (unpaired) resulting from a change in acclimation temperature of rehydrated crabs (compared with partner group control).

† Significant differences (unpaired) between groups of hydrated crabs at the same acclimation temperature.

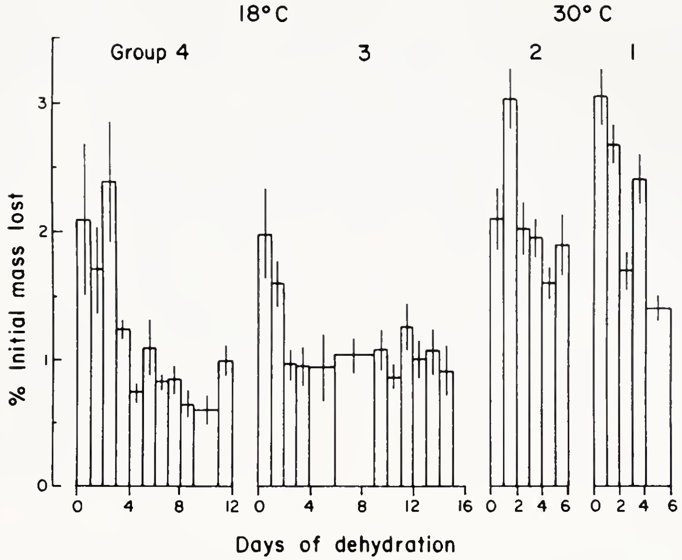

FIGURE 2. Rates of weight lost (presumably as water) with time during dehydration in *C. clypeatus* at 18 and 30°C. Mean ± SEM (n numbers as indicated in legend to Fig. 1).

[Cl$^-$] irrespective of the direction of temperature change and a reduction in ambient temperature also affected circulating [Mg^{2+}] and [K$^+$].

De- and rehydration at constant temperature. In each group mean weight loss declined exponentially with time (Fig. 2). Crabs temperature acclimated prior to dehydration (groups 1 and 3) exhibited the greatest loss on day 1. The settled rate of water loss at 30°C ($\simeq$1.5% initial mass · day^{-1}) was double that occurring at 18°C, and negatively correlated with body mass on a weight specific basis (Fig. 3).

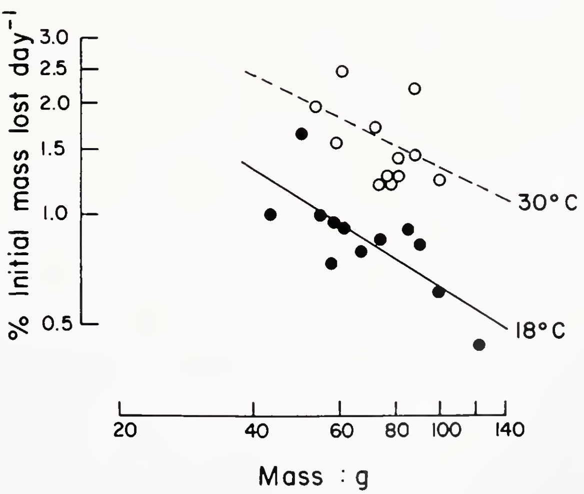

FIGURE 3. The relationship between settled weight specific water loss and weight in *C. clypeatus* at 18 (●) and 30°C (○). Regression equations (on log$_e$ values): 18°C: Y = 3.18 − 0.79X (n = 12, r = 0.76); 30°C: Y = 3.18 − 0.63X (n = 12, r = 0.47).

The cumulative weight lost as water was comparable in each group. Mean weight loss suffered in treatments 1–4 were 12.6 ± 0.7, 12.6 ± 0.8, 15.9 ± 1.9, and 13.6 ± 1.5% initial mass occurring over approximately 6 or 14 days depending on acclimation temperature.

Osmolality and concentrations of all ions (except K^+) were increased as a result of dehydration (Fig. 4a *c.f.* Table II), percentage changes varying for different ions. Ion levels changed consistently at both acclimation temperatures except for Ca^{2+} which exhibited double the elevation at 18°C. These trends were essentially reversed on rehydration (Fig. 4b).

Temperature acclimation in dehydrating crabs. Crabs experiencing dehydration and temperature acclimation concurrently displayed maximum weight loss on day 2–3 (Fig. 2—groups 2 and 4) accompanied as before by significant increases in osmolality and inorganic ion levels (Fig. 5). The increases in osmolality and $[Ca^{2+}]$ were only one half the values reported above (*c.f.* Fig. 4a). The increase in $[Cl^-]$ was considerably in excess of osmolality and Na^+, but was roughly equivalent to the sum of the changes predicted in response to the individual environmental stimuli.

Acid-base balance

Temperature acclimation in hydrated crabs. The characteristic reduction in pH with an increase in acclimation temperature was accompanied by a threefold increase in $P_{,}CO_2$, while $C_{,}CO_2$ was unchanged (Table III). Upon lowering ambient temperature

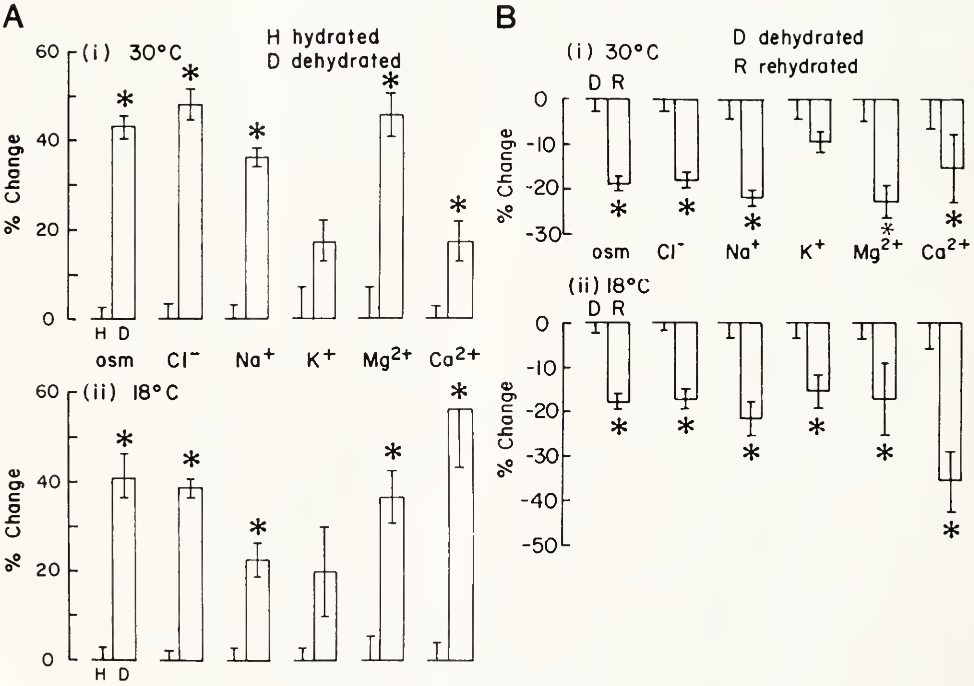

FIGURE 4. Percentile changes in hemolymph osmolality and inorganic ions during dehydration (a) and rehydration (b) at 30°C (i) and 18°C (ii). Asterisks denote significant differences (statistical tests performed on raw data). H, D, and R refer respectively to hydrated, dehydrated, and rehydrated states.

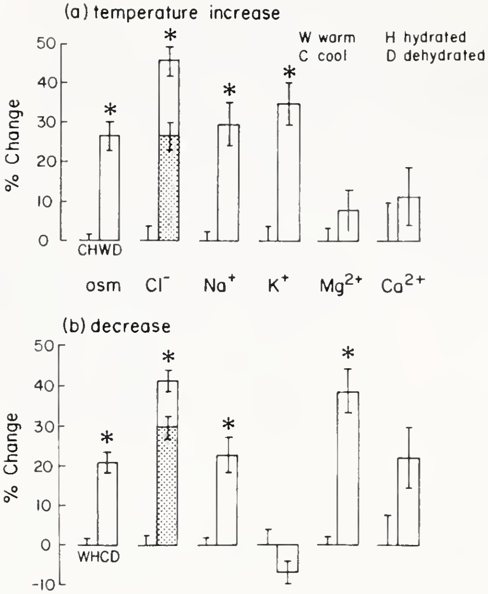

FIGURE 5. Percentile changes in hemolymph osmolality and inorganic ions during dehydration combined with an increase (a) or decrease (b) in ambient temperature. Statistical tests were performed on raw data. W, C, H and D refer respectively to warm, cool, hydrated, and dehydrated states. Stippled area indicates rise in [Cl$^-$] attributable to temperature acclimation.

these trends were reversed; additionally C,CO$_2$, [HCO$_3^-$] and lactate were significantly reduced. As expected, OH$^-$/H$^+$ was maintained constant ($\simeq$10). Coefficients of temperature variation were generally larger with an increase in temperature. Assessment of the origin of this acid-base disturbance on a [HCO$_3^-$] *versus* pH diagram (Fig. 6) indicated that it originated predominantly from respiratory sources (see ΔH$^+$c—Table III) although metabolism contributed significantly especially when temperature was decreasing. In response to an increase in acclimation temperature Δ[Ca^{2+}] and Δ[HCO$_3^-$] increased in the ratio of 2:1 (Table III) but the reverse trend was not evident in the opposite direction of temperature change.

De- and rehydration at constant temperature. Corresponding variations in major determinants of acid-base balance on dehydration (Table IVa; Fig. 7) however, revealed that the accompanying acidosis was possibly attributable to ionic changes (OH$^-$/H$^+$ dropping to $\simeq$4). Moreover Δ[Ca^{2+}] and Δ[HCO$_3^-$] decreased, again in the ratio of 2:1. Converse trends of reduced magnitude were apparent on rehydration (Table IVb; Fig. 7). Significant changes in circulating lactate were only evident at 30°C where a reduction occurred upon dehydration.

Temperature acclimation in dehydrating crabs. In the absence of free water, an increase in temperature (Table Va; Fig. 8) resulted in a hemolymph acidosis attributable mainly to respiration, but less pronounced than the sum of the responses observed to the individual stresses. An additive response was apparent however, in the case of a reduction in acclimation temperature (Table Vb; Fig. 8). In summary, when plotted as a function of acclimation temperature (Fig. 9) it is apparent that the mechanism of pH regulation is the same in both hydrated and dehydrated crabs and is accomplished by an increase in P,CO$_2$ which is characteristic of air-breathing animals.

TABLE III

Variation in major determinants of extracellular acid-base state in hydrated Coenobita clypeatus *acclimated to 18 and 30°C*[1]

		a) Increase in acclimation temperature			b) Decrease in acclimation temperature		
		18°C			30°C		
Variable	Units	x̄	SE(6)	Δ/Δt	x̄	SE(6)	Δ/Δt
pH		7.613	±0.012	−0.019	7.449	±0.028	±0.014*
P,CO_2	torr	6.8	±1.0	+1.337	17.9	±1.5	−0.993*
C,CO_2	mmol l^{-1}	12.6	±1.8	+0.203	14.9	±0.9	−0.446*
HCO_3^-	mmol l^{-1}	12.3	±2.2	+0.160	14.2	±0.9	−0.417*
CO_2	mmol l^{-1}	0.33	±0.05	+0.041	0.65	±0.05	−0.030*
OH^-/H^+		8.6	±0.4	−0.064	11.6	±1.3	−0.159
Lactate	mmol l^{-1}	6.5	±0.4	−0.014	6.4	±0.7	−0.243*
				Δ			Δ
Protein	mg ml^{-1}	125.7	±6.7	−32.5*	113.8	±9.8	−14.7
β	mmol l^{-1} pH $unit^{-1}$	18.3	±0.9	−4.5*	16.6	±1.4	−2.0
pK'_1		6.06	±0.03	+0.08	6.11	±0.06	+0.01
ΔH^+m	m-equiv l^{-1}			+0.9 ± 1.4			−2.2 ± 1.0
ΔH^+c	m-equiv l^{-1}			+4.2 ± 0.6			−3.0 ± 0.7
$\Delta[Ca^{2+}]$	m-equiv l^{-1}			+4.8 ± 2.1			+2.2 ± 2.7
$\Delta[HCO_3^-]$	m-equiv l^{-1}			+2.5 ± 2.8			−3.7 ± 1.7

[1] The effect of a) increase and b) decrease in acclimation temperature. Asterisks beside coefficient of temperature variation denote that values changed significantly on temperature acclimation. Consult text for calculation of derived variables.

DISCUSSION

Temperature acclimation of hydrated crabs

The present investigation suggested that water economy was controlled to some extent in *C. clypeatus* (Table I). Crabs transferring to more capacious shells did not load extra water and there was no evidence of overhydration when water became available again after deprivation. The loss of ≃2.5 g of total mass on acclimation to 30°C probably represents water loss from the shell reservoir, arising unavoidably from heightened activity at increased temperature. The reduction in mass after sampling may be similarly attributed to shell water spillage since it was greater at 18°C.

With free access to 10% sea water in the present study *C. clypeatus* maintained the hemolymph slightly hypo-osmotic and hypoionic compared with full strength sea water (Table II—*c.f.* Gross, 1955; de Wilde, 1973). Thermal acclimation did not consistently affect these levels except for Cl^-. The increase following a rise in acclimation temperature may result from exchange for endogenous bicarbonate as a mechanism for regulating pH (see Randall and Cameron, 1973, and below). Less easily explained is the increase in Cl^- upon cooling which reaffirms our poor understanding of Cl^- regulation in the decapods (Gross, 1963; Burggren and McMahon, 1981). The significant change in Mg^{2+} and K^+ in group 3 presently cannot be explained.

Values reported for pH, P,CO_2, and [HCO_3^-] in *C. clypeatus* (Table III) were generally similar to those of other air-breathing decapods at comparable temperatures (Cameron and Mecklenburg, 1973; Smatresk *et al.*, 1979; Wood and Randall, 1981).

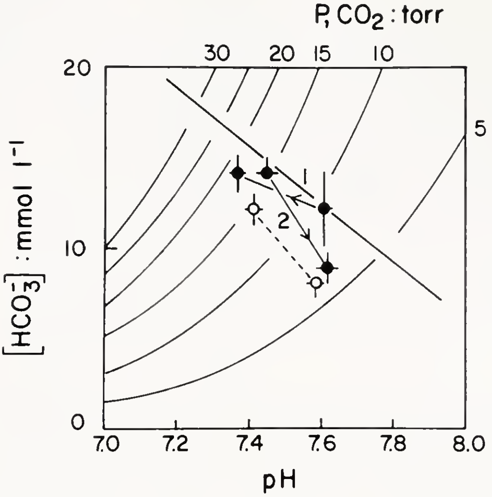

FIGURE 6. $[HCO_3^-]$ *versus* pH diagram indicating changes in acid-base status in prebranchial hemolymph of *Coenobita clypeatus* during temperature acclimation of hydrated animals. 1 and 2 indicate temperature increase and decrease respectively. Open symbols indicate acid-base status of rehydrated animals at corresponding acclimation temperatures. P,CO_2 isopleths were constructed using mean values of 6.102 for pK'_1 and 0.0425 mmole l^{-1} $torr^{-1}$ for α. For graphical purposes the average slope of the CO_2 buffer line is indicated.

An OH^-/H^+ ratio of ≃9 is close to values reported by Truchot (1973) for the intertidal shore crab *Carcinus* (≃12). Since values for aquatic species are around 16–25 (Howell *et al.*, 1973) it would appear that relative alkalinity decreases with reliance on air-

TABLE IV

Variation in major determinants of extracellular acid-base balance in Coenobita clypeatus *as a result of (a) dehydration and (b) rehydration at 18 and 30°C*[1]

	(a) Dehydration		(b) Rehydration	
Variable	18°C Δ	30°C Δ	18°C Δ	30°C Δ
pH	−0.118*	−0.189*	+0.055	+0.055
P,CO_2	+3.4	−3.1	−2.0*	−4.5
C,CO_2	+5.8*	+1.2	−2.9*	−1.8
HCO_3^-	+4.5	+1.3	−2.8	−1.7
CO_2	+0.15	−0.15	−0.09*	−0.12
OH^-/H^+	−4.0*	−4.4*	+2.1	+2.3
Lactate	−1.6	−5.2*	+0.7	+1.6*
Protein	−16.8	−16.9	−7.7	−9.7
β	−2.3	−2.4	−1.1	−1.4
pK'_1	−0.08	−0.30*	+0.03	+0.05
ΔH^+m	+2.7 ± 0.2	+1.6 ± 0.2	−1.8 ± 1.3	−0.8 ± 0.8
ΔH^+c	+2.1 ± 0.5	−0.5 ± 0.6	−1.3 ± 0.4	−0.9 ± 0.2
$\Delta[Ca^{2+}]$	−8.0 ± 3.4	−18.1 ± 1.2	−1.3 ± 2.3	+5.9 ± 3.3
$\Delta[HCO_3^-]$	−2.9 ± 0.4	−9.8 ± 1.0	+0.9 ± 1.2	+3.8 ± 1.0

[1] Units and symbols—as for Table III.

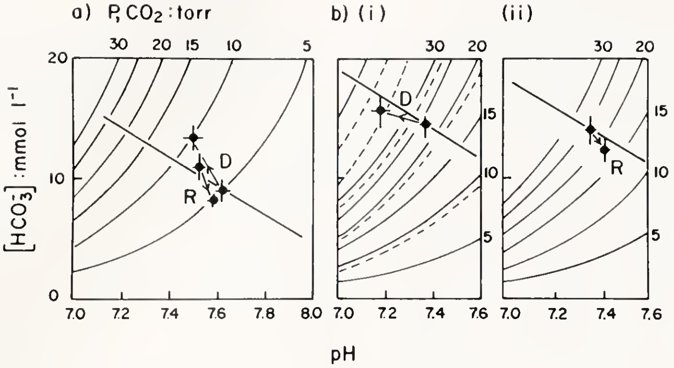

FIGURE 7. Changes in acid-base status in prebranchial hemolymph of *Coenobita clypeatus* during dehydration (D) to 86% of initial mass and subsequent rehydration (R) at 18°C (a) and 30°C [b (i) and (ii)]. At 18°C, P,CO_2 isopleths were constructed using mean values of 6.045 for pK'_1 and 0.048 mmole l^{-1} $torr^{-1}$ for αCO_2. In b (ii) mean values employed were 6.120 and 0.035 mmole l^{-1} $torr^{-1}$ respectively. At 30°C there were significant differences between operational pK'_1 for hydrated and dehydrated crabs. In b (i) solid isopleths correspond to hydrated crabs ($pK'_1 = 6.135$, $\alpha = 0.036$ mmole l^{-1} $torr^{-1}$) and broken isopleths to dehydrated crabs ($pK'_1 = 5.837$ and $\alpha = 0.034$ mmole l^{-1} $torr^{-1}$).

breathing as in ectothermic vertebrates (Howell *et al.*, 1970). A discrepancy between measured P,CO_2 values and those calculated tâking pK'_1 from Truchot's (1976a) nomographs for *Carcinus* (interpolated as 6.06 and 5.99 respectively at 18 and 30°C in the hydrated state) probably relates to a higher value for β (see Methods) in the terrestrial species (*c.f.*, McMahon *et al.*, 1978; Smatresk *et al.*, 1979). This explains

TABLE V

Variation in major determinants of extracellular acid-base balance in Coenobita clypeatus *on dehydration combined with a) increase and b) decrease in acclimation temperature*[1]

	a) Increased acclimation temperature		b) Decreased acclimation temperature	
Variable	Δ	Δ/Δt	Δ	Δ/Δt
pH	−0.249* (−0.429)	−0.020	+0.082* (+0.053)	+0.007
P,CO_2	+15.9* (+13.6)	+1.275	−10.8* (−9.0)	−0.862
C,CO_2	+2.1 (+3.7)	+0.168	−3.5* (+0.3)	−0.276
HCO_3^-	+1.6 (+3.3)	+0.130	−3.2 (+9.7)	−0.252
CO_2	+0.44* (+0.36)	+0.035	−0.31* (−0.23)	−0.025
OH^-/H^+	−1.0 (−5.2)	−0.080	−5.4* (−6.0)	−0.432
Lactate	−4.4* (−5.4)	−0.355	−2.2* (−4.6)	−0.178
Protein	−29.3*		−21.7	
β	−4.1*		−3.0	
pK'_1	+0.04		−0.09	
ΔH^+m	−1.2 (+2.5)		−1.5 (+0.5)	
ΔH^+c	+2.9 (+3.7)		−1.6 (−0.9)	
$\Delta[Ca^{2+}]$	−8.9 (−13.3)		−4.9 (−5.8)	
$\Delta[HCO_3^-]$	−4.3 (−7.3)		−8.4 (−6.6)	

[1] Values in parentheses are those which can be predicted from addition of responses to individual stimuli. Units and symbols—as for Table III.

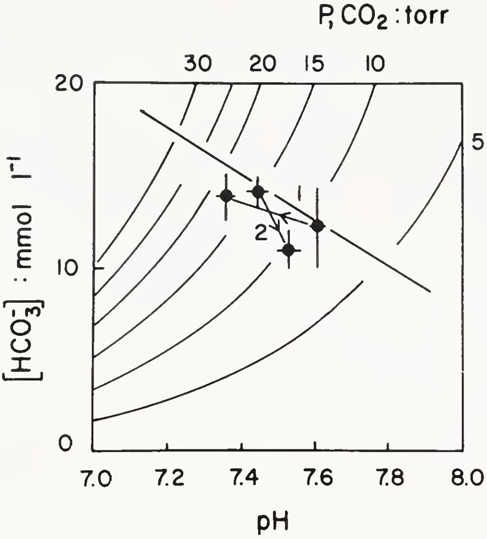

FIGURE 8. Changes in acid-base status in prebranchial hemolymph of *Coenobita clypeatus* on dehydration combined with acclimation to (1) increasing and (2) decreasing ambient temperatures. The mean values for pK$'_1$ and αCO_2 used in the construction of CO_2 isopleths were 6.087 and 0.0415 mmole l^{-1} torr^{-1} respectively.

why P,CO_2 values presently measured were higher than those calculated using these constants in *C. brevimanus* in an earlier study (McMahon and Burggren, 1979). An alternative explanation could reside in their separate distribution in the natural environment, where *C. clypeatus* occurs further from the sea (Borrodaile, 1903; Bright, 1966). Resting lactate levels were higher than in aquatic species (*e.g.*, Booth *et al.*, 1982) and may be associated with sustained tonic activity in cheliped muscles associated with closure of the shell operculum.

The present data for temperature-dependent pH regulation in *Coenobita* (Table III; Fig. 6) produced ΔpH/Δt values characteristic of other ectotherms (Rahn, 1966). The similarity of these to ΔpK/Δt of various intracellular buffers is now thought to be more complex than originally envisioned in the "alphastat" theory advanced by Reeves (1972; see Cameron and Kormanik, 1982). Other authors (*e.g.*, Ackerman and White, 1980) have investigated the dependence of ΔpH/Δt on the direction of temperature change with similar results to the present study.

The most important single finding of the present investigation was that pH was controlled by the ventilatory regulation of P,CO_2, irrespective of the availability to the animal of free water and thus the provision of an aquatic route for HCO_3^- exchange (Fig. 9). Most air-breathing species do not have simultaneous contact with water. Therefore circulating $[HCO_3^-]$ is generally maintained during an increase in temperature whereas a decrease is observed in aquatic species (Cameron and Batterton, 1978; McMahon *et al.*, 1978). In animals where both mechanisms operate simultaneously (*e.g.*, the turtle—Howell *et al.*, 1970 or shore crab—Truchot, 1973), a decrease in $[HCO_3^-]$ generally accompanies a rise in P,CO_2.

The increase in $[HCO_3^-]$ in *Coenobita* was therefore largely unexpected (Fig. 9), although Heisler *et al.* (1980) made a similar observation in dogfish. In the present case it may simply reflect a shift to increasingly aerial routes of gas exchange at increased acclimation temperature which would be characterized by elevated $[HCO_3^-]$.

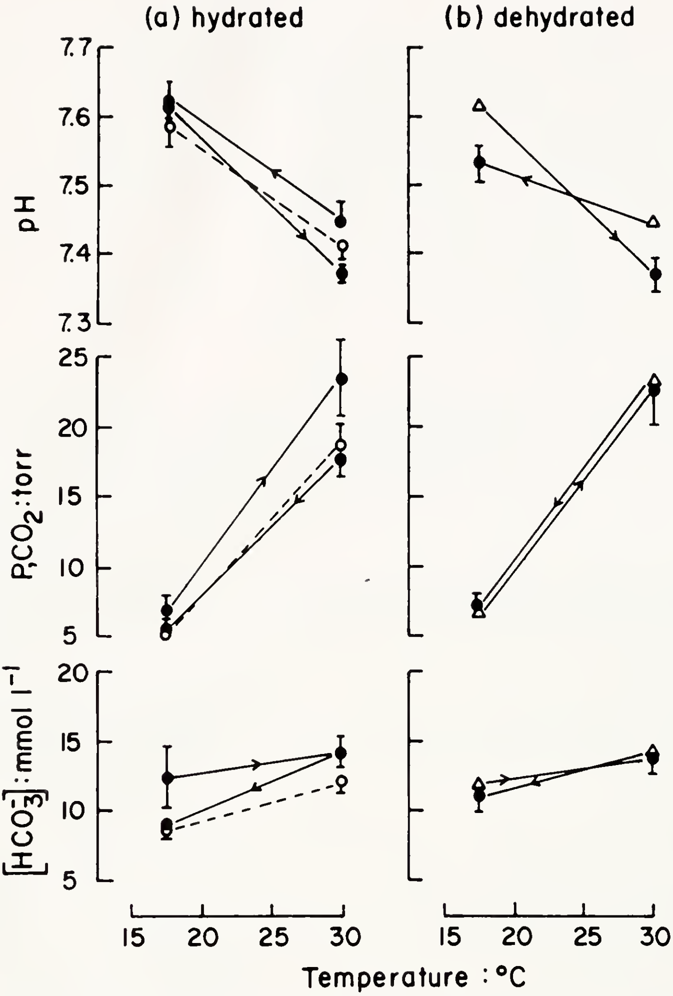

FIGURE 9. Variation in hemolymph pH, P,CO_2, and $[HCO_3^-]$ with temperature in *Coenobita clypeatus* in the presence (a) and absence (b) of free water. Paired sample means and SE are joined together with an arrow indicating the direction in which the temperature change was made. Open symbols are values for rehydrated animals (unpaired). In (b) values are compared with corresponding control group (*i.e.*, hydrated animals at original acclimation temperature—designated by triangular symbols).

This is certainly true of other bimodal breathers such as shore crabs (Taylor and Wheatly, 1979) and adult salamanders (Burggren and Wood, 1981). The increase in HCO_3^- may compensate for an overshoot in P,CO_2 which, otherwise occurring, would double the pH disturbance. Figure 10 illustrates this point indicating the contribution of different variables to the observed change in extracellular pH (calculated by changing one variable in the Henderson-Hasselbalch equation).

The $[HCO_3^-]$ which accumulates in the hemolymph during a temperature increase could originate from metabolic CO_2 or another fluid compartment *e.g.*, intra- to extracellular transfer (Heisler, 1978). In this respect exchange of Cl^- for endogenous bicarbonate could be one mechanism involved. Alternatively, HCO_3^- may be mobilized

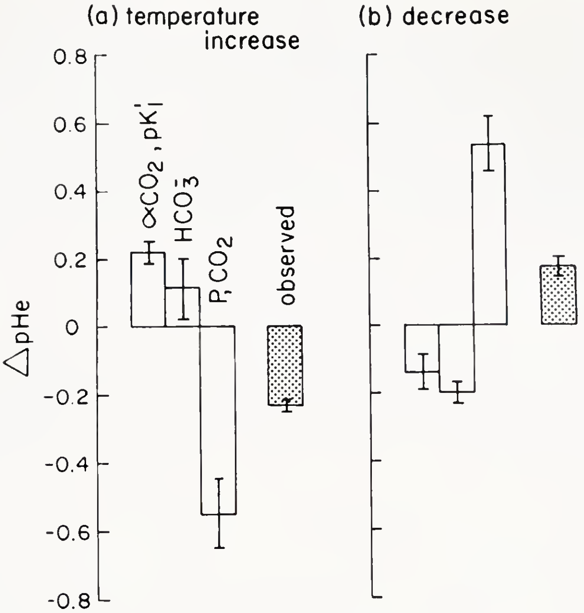

FIGURE 10. Contribution of changes in individual parameters to the relationship between pH and temperature found experimentally (stippled) in *Coenobita clypeatus* on (a) increase and (b) decrease in acclimation temperature. The label indicates the parameter which was varied.

from exoskeletal $CaCO_3$ since a disproportionate increase in hemolymph Ca^{2+} was detected (Table III). If this is so, it constitutes further evidence of "shell buffering" of hypercapnic acidosis in terrestrial species (see deFur *et al.,* 1980; Henry *et al.,* 1981; Wood and Randall, 1981) although one would expect a Ca^{2+}:base excess of 1:1 if $CaCO_3$ is the source of the buffering (Henry *et al.,* 1981).

De- and rehydration at constant temperature

Since the 14% reduction in total mass suffered by *Coenobita* included body and shell water, comparison with dehydration tolerance of other species is difficult. Nonetheless the efficacy of the adopted shell in reducing evaporation becomes apparent when comparing *settled* rates of water loss. Values of 0.7 and 1.4% initial mass day^{-1} at 18 and 30°C are a fraction of levels in both aquatic ($\simeq$12% BW day^{-1}—Herreid, 1969) and terrestrial species (*e.g.,* 5.5% in *Gecarcinus*—Bliss, 1966; 15.1% in *Ocypode*—Lutz, 1969). When deprived of water, *Coenobita* remained continuously retracted into the shell, the major chela and flattened ambulatories serving ostensibly as a reasonably water-tight operculum (see Harms, 1929; Magnus, 1960). 12% loss in body weight is generally lethal in aquatic brachyurans (Dandy and Ewer, 1961), extending upwards of 25% in inland species such as *Holthuisana* which has an exceptional ability to withstand desiccation (Greenaway and Macmillen, 1978). Interruptions to the exponential loss of water (as in Fig. 2) may arise from shell water spillage occasioned by locomotion or voluntary release due to build up of toxic wastes (A. W. Pinder and B. R. McMahon, unpub.). Loss of shell water is apparent when crabs are kept on dry sand.

The exponential decrease in rate of water loss with mass (Fig. 3) parallels other studies (Schmidt-Nielsen, 1964; Herreid, 1969) implicating scale functions; however the slope presently reported exceeds those relating to surface area:volume (−0.33) or metabolism (−0.15; Scholander *et al.,* 1953). Water loss in the hermit crab may therefore be a complex function of the surface area of the shell water to air interface. Rate of evaporative loss did not correlate with any dimension of the molluscan shell nor shell/animal association index (see Wheatly, 1984) and therefore refutes the idea of an optimal "shell fit" although we think this possibility should be pursued.

During dehydration the increase in hemolymph osmolality (Fig. 4a) suggests that some water was lost from the extracellular compartment. Disproportionate increases in hemolymph electrolyte concentrations indicated active ionoregulation with other fluid compartments at this time. Paradoxically, in the earlier study on *C. brevimanus,* (Burggren and McMahon, 1981), ion levels were maintained despite a marked increase in osmolality which presumably involved organic osmolytes such as free amino acids increasing in response to the hyperosmotic condition developing in the blood.

The hemolymph acidosis during dehydration was possibly attributable to a shift in the ratio of cations to anions (Table IVa; Fig. 7). However the acid-base disturbance resulting from respiratory causes was greater than previously reported (Burggren and McMahon, 1981). It remains to be determined whether the increase in $P_{,}CO_2$ relates to loss of an aquatic route for CO_2 excretion as shell water evaporates or to hypoventilation in order to minimize water lost in the respiratory stream (Taylor and Wheatly, 1981). The parallel disappearance of HCO_3^- and Ca^{2+} from the hemolymph during dehydration (Table IVa) suggests deposition of $CaCO_3$. In the absence of an external source of HCO_3^-, the CO_3^{2-} store could have originated from retention of respiratory CO_2 (see Wood and Randall, 1981).

The reappearance of lactate on rehydration (Table IVb) would suggest that it was sequestered intracellularly during dehydration and a similar conclusion can be made for K^+. On the whole, the ionic events occurring on rehydration were more uniform (Fig. IVb; *c.f.* IVa), suggesting a more passive physiological response.

Temperature acclimation in dehydrating crabs

Although total water loss was identical in all dehydration treatments, animals simultaneously undergoing a change in acclimation temperature exhibited less pronounced changes in circulating ion levels (Fig. 5). This could be explained if blood volume was maintained at the increased expense of shell and tissue fluid, although we have no evidence to support this. Lutz (1969) observed a similar strategy under severe dehydration (≃25% BW) in *Sudanonautes.* Furthermore temperature may alter the distribution of body water. Since Cl^- exhibited a disproportionately large increase in response to the combined stimulus (Fig. 5), one would expect this anion excess to reduce the strong ion difference (Stewart, 1978—SID calculated as $[Na^+] + [K^+] + [Ca^{2+}] + [Mg^{2+}] - [Cl^-] - [HCO_3^-]$). Yet a significant acidosis only occurred on warming, from which one can assume that ions not presently measured contributed to the SID.

Coenobita regulated hemolymph pH in the face of temperature change by ventilatory control of $P_{,}CO_2$ irrespective of its relative state of hydration (Fig. 9). Truchot (1973) similarly found that ventilatory control mechanisms were predominant in the intertidal shore crab in both air and water. An increase in acclimation temperature, however, did lessen the acid-base disturbance resulting from dehydration (Table V; Fig. 6) which has ecophysiological relevance since these two parameters will frequently change together in the environment. The reverse situation, *i.e.,* dehydration combined with a reduction in temperature, would rarely occur in nature unless animals were washed ashore by warm currents in subtropical areas (de Wilde, 1973); it is not surprising that the stimuli show no physiological interaction.

In summary, the present investigation suggests that loss to circulating volume in dehydrated hermit crabs is minimized by simultaneous exposure to temperature variation which, in poikilotherms, elicits a number of physiological responses. This attempt to regulate the composition of extracellular fluid exemplifies hierarchical homeostatic regulation in response to combined environmental stimuli. Although a reservoir of water is conveyed by this species into the aerial environment, it is not essential for pH regulation during temperature variation, which continues in a fashion characteristic of other air-breathers. The physiological significance of the shell water remains as yet undetermined.

ACKNOWLEDGMENTS

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PATTERN REGULATION IN AUTOGRAFTS OF *HYDRA OLIGACTIS*: POSITIONAL VALUES AND DIFFUSABLE MORPHOGENS

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ABSTRACT

Grafted *Hydra oligactis* are employed in this study in an attempt to determine if head regeneration in autografts of the species conforms to a positional-information-type control. Two groups of hydras are used: one group has the gastric regions reversed (g-reversal), and a second group with gastric regions and budding regions reversed (gbr-reversal). Regeneration of secondary (2°) heads in the original subhypostomal region of the reversed body regions is observed for both groups at 48, 72, and 96 hours post-grafting. Secondary heads occur in both groups with a significantly greater frequency of 2° heads forming in the gbr-reversal group. These results concur with those seen in "multiply-grafted" hydras wherein tandemly arranged gastric regions are inserted into the grafted animal. Namely, 2° head formation is more frequent at borders located progressively farther from the terminal head. The findings of this study argue strongly for the occurrence of an inhibitory signal (positional information signal) being superimposed upon the graded positional values (for head formation) along the hydra's body column. Furthermore, this study demonstrates that the occurrence of similar results in "multiply-grafted" hydras is a function of intrinsic controls of pattern formation and not an artifactual consequence of abnormal elongation of the animals.

INTRODUCTION

Development of complex organisms with well defined patterns of morphological structure from the modest beginnings of the zygote has long intrigued developmental biologists. Many of the recent models put forth to explain this phenomenon incorporate the concept of cells being endowed with the ability to recognize their neighbors, and to know their position within a tissue field (Wolpert, 1971; Bryant, 1974; French *et al.*, 1976). This type of communication, and subsequent cell-cell interactions render cells non-equivalent and thus programs different developmental commitments by the cells. This concept is termed positional value.

Two of the more popular models purporing to explain the control of pattern formation, namely those of Vernon French and Louis Wolpert, both employ the concept of positional value. French's "polar coordinates" model proposes (French *et al.*, 1976) that cells have positional values which are defined by the cell's position along the circumference of a circle and its position on the radius of that circle. French's model states that regeneration within the circumference of the circle is by intercalary growth between apposing abnormal neighbors. The shortest intercalary route would always be taken during regeneration, even if it means producing a mirror image rather than replacing the missing parts. When all cells have normal neighbors regeneration would cease.

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Wolpert's model (Wolpert, 1971) talks about positional values along some axis, with an inhibitory signal (positional information signal) superimposed upon the positional values. The positional information signal would suppress specific morphogenetic events (such as head regeneration in *Hydra*) by cells that would otherwise perform these acts.

The occurrence of the diffusable inhibitor of head formation, which exists as a gradient decreasing in the direction of the foot, was demonstrated in multiply-grafted *Hydra viridis* (Shostak, 1972, 1973; Shostak and Adams, 1975). Significantly greater frequencies for secondary (2°) head regeneration were observed at graft borders located progressively farther from the terminal head in multiply-grafted animals with three tandemly arranged gastric regions (3g hydras). Since each of the three gastric regions possess theoretically equal morphogenetic competency the gradient in 2° head regeneration was interpreted as resulting from a parallel gradient of the "head inhibitor." Wolpert's model incorporates such an inhibitory substance, existing as a gradient due to "source-sink" activity (Crick, 1970) as depicted in Figure 1. The positional values for *Hydra* would result in a graded disposition toward hypostome and tentacle formation (also depicted in Fig. 1). Cells closer to the head would be more disposed toward head formation.

For the purpose of this study the body regions of *Hydra* have been subdivided into arbitrarily assigned positions representing the relative distance from the head (H). Autografts of *H. oligactis* are employed in this study in an attempt to determine if regeneration of 2° heads conforms to a positional-information-type control.

MATERIALS AND METHODS

H. oligactis were mass cultured in 20 cm diameter Pyrex dishes in an incubator at 19 ± 0.2°C. The hydras were fed *Artemia salina* nauplii daily and the medium

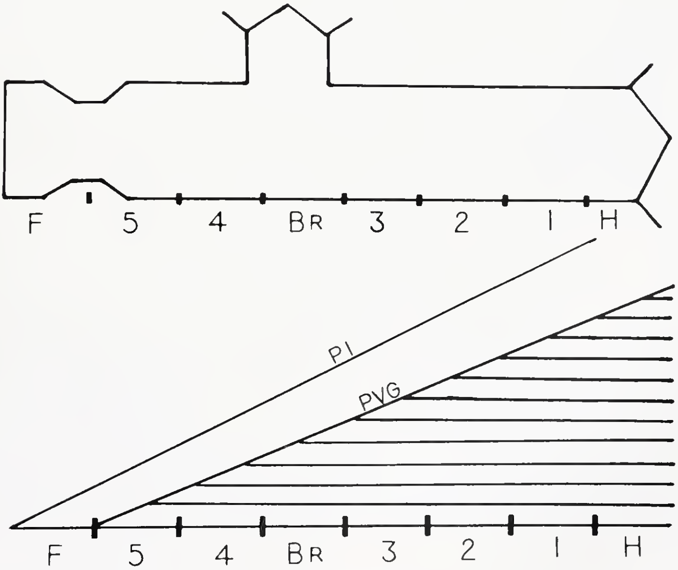

FIGURE 1. Diagram showing the positional value gradient (PVG) and arbitrarily assigned positions H–F for *H. oligactis*. Superimposed upon the positional value gradient is a positional information (inhibitor) gradient (PI).

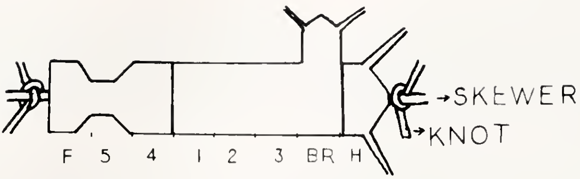

FIGURE 2. Diagram showing an animal that has been transected at the positions 1/H border and the positions Br/4 border followed by reversal of the gastric and budding regions (gbr-reversal) and threading of the graft pieces on a skewer of human hair. Two knots of human hair have been placed at either end of the graft pieces to insure apposition during healing.

(artificial pond water, Loomis and Lenhoff, 1956) was changed approximately one hour after feeding to remove uningested nauplii. Experimental animals were kept in 15 mm × 60 mm Petri dishes, one animal per dish, to allow for individual observations of each animal and to prevent any chance of confusing 2° heads with retained buds. All animals used in this study had a single stage I bud (bud staging methods of Shostak *et al.,* 1968 were used).

Grafting was performed by first making the appropriate transections and placing the graft pieces on straight skewers of human hair (Shostak, 1972). Pre-tied knots of human hair were loosened with watchmaker's forceps and placed on each end of the skewer. The knots were then tightened and slid along the skewer until all cut surfaces were touching (Fig. 2). Grafts were allowed to heal for two and one-half hours and were then removed from the skewer. Secondary head regeneration was monitored at 24 hour intervals for a period of four days. Any differences between the frequencies of 2° head regeneration in the groups of animals were tested using Chi square at a 95% confidence level.

RESULTS AND DISCUSSION

Regeneration of 2° heads at the distal end of position 1 is observed at 24, 48, 72, and 96 hours post-grafting. The resulting observations are shown in Table I. No 2° head regeneration is seen at 24 hours for either group of animals, thus 24 hour data are not shown. At 48 hours 18% of the animals in the g-reversal group have

TABLE I

Head regeneration in Hydra oligactis *following reversal grafting*

Treatment	48 Hours	72 Hours	96 Hours
	F, %	F, %	F, %
Gastric region reversal n = 39	7, 18	8, 21	8, 21
Budding region and gastric region reversal n = 39	26, 67 .001 > *P*	28, 72 .001 > *P*	28, 72 .001 > *P*

F: frequency; *P*: probability of chance deviation between control and experimental frequence (X^2).

regenerated 2° heads. By 72 hours 21% of these animals have regenerated 2° heads and the percentage remains the same at 96 hours. The gbr-reversal group shows 2° head regeneration percentages of 67, 72, and 72% at 48, 72, and 96 hours respectively. The frequency of 2° head regeneration in the gbr-reversal group is higher than that for the g-reversal group at all three time intervals shown in Table I. Figure 3 shows an example of an animal with a 2° head and also a retained bud. This example is chosen to illustrate the point that both due to the relative position and polarity of each, as well as the individual data taken on each animal, 2° heads and retained buds are easily distinguishable. Thus all instances of 2° heads reported here are unquestionably head regenerates.

What the effects of g-reversal and gbr-reversal would be on the relationship between the diffusable inhibitory gradient and the regional positional values along the body column of hydra is seen in Figures 4a and b. Either of the reversal techniques could give the positional value of region one an advantage over the effects of the inhibitory signal thus presenting the possibility of 2° head regeneration. When both the gastric and budding regions are reversed the advantage given the positional value of region one is even greater thus predicting the greater frequency of 2° head regenerates observed for this group.

The results of this study argue strongly for the existence of a head inhibitory signal existing as a gradient along the oral-aboral axis of *Hydra* like that proposed by Shostak (1973) and Wolpert (1969). This study also supports the concept that this head inhibitory gradient is superimposed upon a graded positional value for head formation. Interaction between this position value for head formation and the head inhibitor then are responsible for controlling the pattern of head formation in *Hydra*.

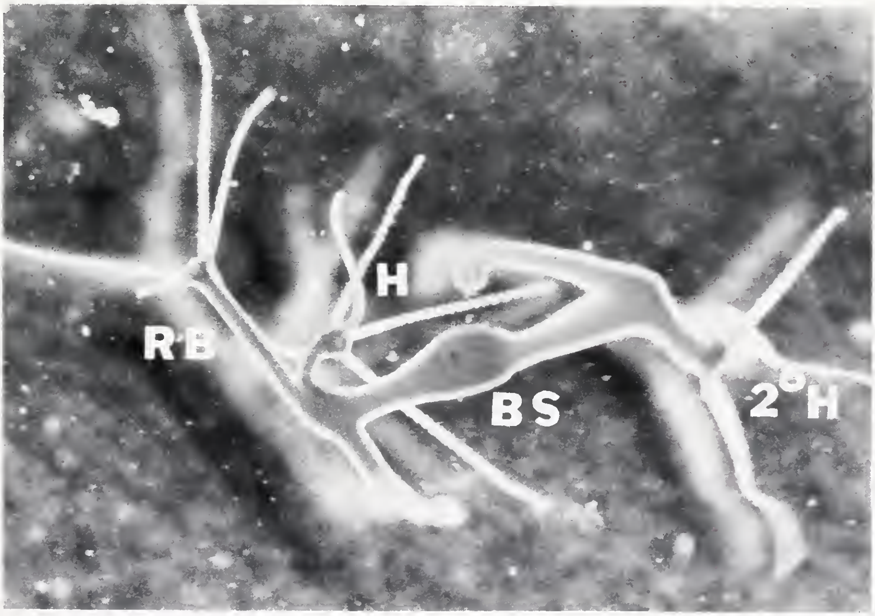

FIGURE 3. This gbr-reversal grafted animal shows a 2° head (2°H), a retained bud (RB), the terminal head of the original animal (H), and the body stalk (BS) of the original animal.

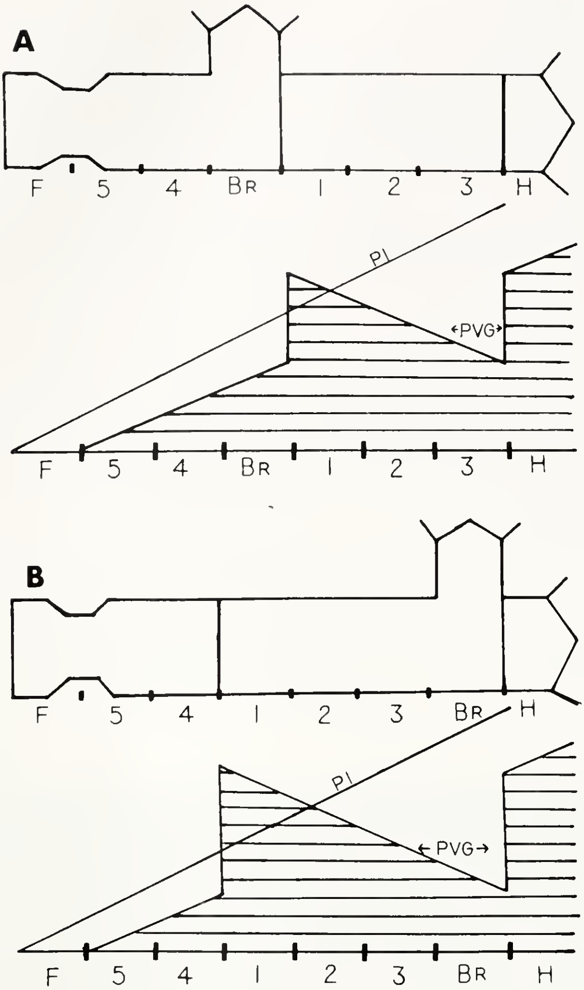

FIGURE 4. Diagrams showing the positional values at points along the hydranth following reversal grafting. Note the greater advantage that position 1 has over the inhibitory signal (positional information) when gbr-reversal is performed (4b) as compared to g-reversal (4a).

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H1 HISTONES OF ADULT TISSUES OF THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*

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ABSTRACT

Embryonic development of *Strongylocentrotus purpuratus* is characterized by a shift from a faster moving H1 (H1m) found during cleavage stages to a slower moving H1 (H1g) which is synthesized during the blastula and later developmental stages. Sperm have been characterized as having still other H1 histones. The present work describes the H1 histones of adult tissues (coelomocytes, tube feet, intestine, mesentery, testis, and sperm), as revealed by electrophoresis of histone samples in polyacrylamide gels containing sodium dodecylsulfate (SDS). Indications are that sperm possess two subtypes not found in somatic tissues or embryos and that adult tissues possess one H1 subtype not found in sperm or embryos. The possibility that these H1 subtypes represent post-translational modification is discussed.

INTRODUCTION

Shifts in the expression of histone H1 gene subtypes are not the result of post-translational modification of the proteins, but are the result of the expression of different sets of histone H1 genes (Ruderman and Gross, 1974; Kunkel and Weinberg, 1978; Hohman, 1980; Harrison and Wilt, 1982). In considering mechanisms of control of histone gene expression, it is of some consequence to be aware of the number of H1 subtype proteins encoded by histone genes which are expressed at different times during development or which are expressed in the adult as tissue specific H1 subtypes. In the sea urchin, the embryos have been far more thoroughly studied than the adults. Brandt *et al.* (1979) describe the H1 components of the adult gut of the sea urchin *Parechinus angulosus.* To date, there are no data available dealing with the H1 histones of the various adult tissues of *S. purpuratus,* though the genes coding for the embryonic histones of this animal have been extensively studied (Cohen *et al.,* 1975; Newrock *et al.,* 1978; Kedes and Maxson, 1981; Maxson *et al.,* 1983).

The investigations reported here deal with the H1 histones of the intestine, tube feet, mesentery, coelomocytes, testis, and sperm of *S. purpuratus.* Histones of plutei and blastulae are included for comparison.

MATERIALS AND METHODS

Adult *Strongylocentrotus purpuratus* were obtained from live tanks at the Friday Harbor Laboratories of the University of Washington. Adult tissues were dissected from animals whose coelomic fluid had been previously drained using a 50 cc syringe

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fitted with an 18 ga. needle. Intestine and mesentery were distinguished by their pigmentation; mesentery is deep purple, intestine is lighter brown. Tube feet were obtained by cutting out those sections of the test containing the ambulacral system, and scraping the inner surface to remove the inner portions of tube feet. Coelmic fluid was centrifuged to recover the coelomocytes. Testis was obtained as free as possible of spermatozoa by prior KCl injection to induce spawning, and by repeated washing to remove sperm. It proved to be virtually impossible to prepare testis free of contaminating sperm. Sperm cells were also collected for histone extraction. All tissues were washed in cold filtered sea water to remove parasitic ciliates prior to homogenization. Ovary was initially included in the list of tissues to be examined. After processing, it was found that the sample obtained from ovary contained too many contaminating basic proteins, and too little histone to provide a useful comparison with other tissues.

Embryonic stages were obtained by standard fertilization and culture techniques following KCl injection to induce spawning. Histones were extracted from early blastulae and from pluteus larvae.

Following washing, tissues or embryos were washed in 2 changes of a medium containing 0.45 *M* NaCl, 0.025 *M* EDTA, pH 7.4, and 2 changes of a second medium containing 0.075 *M* NaCl, 0.01 *M* EDTA, 0.01 *M* Tris, 0.1 *M* $NaHSO_3$ (TENN). Twenty microliters of TX100 were added per 100 ml of TENN. Tissues and embryos were homogenized in 5 volumes of TENN for 15 seconds in a Waring blender (50 ml cup). The homogenates were examined for unbroken cells using phase contrast optics, and then centrifuged at 2000 rpm for 10 minutes to sediment nuclei. The nuclear pellet was suspended in 2 to 5 ml of TENN, layered over 2 volumes of 1 *M* sucrose in TENN, and centrifuged for 10 minutes at 5000 × *g* in a Sorvall HB4 rotor at 4°C. The nuclear pellet was suspended in 1–2 ml of distilled water, and extracted with an equal volume of 1 *N* H_2SO_4 for 2 hours, in an ice bath. Histones were precipitated from the supernatant with 2.5 volumes of ethanol overnight, and dissolved in 0.5–1.0 ml of 2% Sodium dodecylsulfate (SDS), 0.0375 *M* Tris, 4 *M* urea, 5% 2-mercaptoethanol.

Electrophoresis was performed using 13.5% SDS-acrylamide gels (75:1 bis acrylamide), and a 4% (75:1) spacer gel (Laemmli, 1970). The electrode buffer consisted of 0.025 *M* Tris, 0.19 *M* glycine, pH 8.4, and 0.1% SDS. Bromphenol blue in 20% sucrose was used as a tracking dye. Gels were run at constant power of 5–6 W (80 V, 75 mA), and were stained with Coomassie brilliant blue R.

RESULTS

Figure 1 shows the resolution of H1 histones of embryonic and adult tissues. The faster moving H2–H4 histones have been intentionally electrophoresed out of the gel in advance of the bromphenol blue front, which remains as an index for estimation of distance migrated by the H1 histones. This procedure permitted migration over a greater distance, with greater resolution of the H1 bands. The extraction procedure removed core histones from isolated nuclei with variable results, especially for H4. The R_f values in Table I are derived from the migration pattern in Figure 1.

Adult tissues all show the same two H1 bands, the slower of which has the same R_f value as embryonic histone H1g. The more rapidly migrating Adult H1-II histone appears distinct from embryonic H1 histones and sperm H1 histones. Testis and sperm show two (slower) H1 bands which are distinct from embryonic H1's and other tissue H1 bands.

1

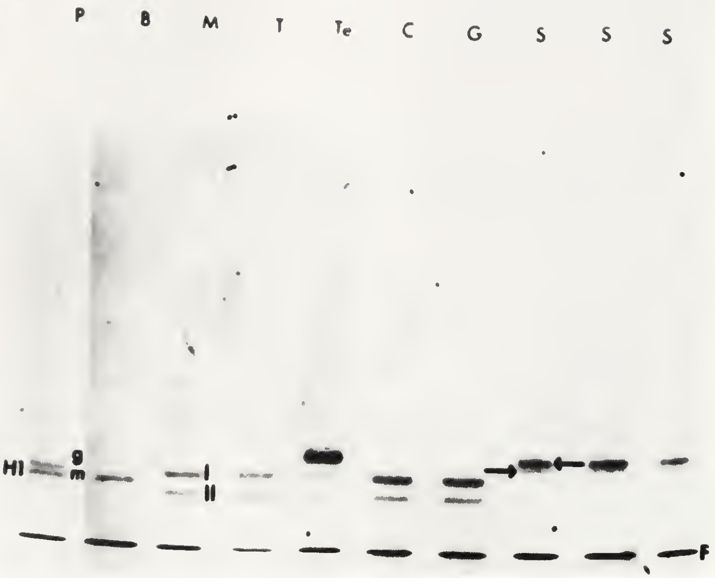

FIGURE 1. Lanes contain 20 μl samples of embryonic stages or adult tissues: Lane 1, P = pluteus. 2, B = blastula. 3, M = mesentery. 4, T = tube feet. 5, Te = testis. 6, C = coelomocyte. 7, G = intestine. 8–10, S = sperm. H1, g & m indicates position of embryonic histones. I and II = positions of adult H1 histones. Arrows indicate H1 histones of sperm. F = bromphenol blue front. Anode at bottom of gel.

DISCUSSION

Some evidence exists to support the notion that the expression of the H1 genes coding for the various subtypes is related to factors regulating cell phenotype in mammalian and sea urchin systems (Hohman, 1980; Arceci and Gross, 1981). For this reason, attention has been focused on H1 histones, as opposed to the more highly conserved core histones.

The present study indicates that at least one H1 variant (subtype) different from those found during embryonic development may be present in adult somatic tissues, and that sperm possess two subtypes not found in embryos or adult tissue. It remains possible that adult H1-II represents a phosphorylated modification of other histones resolved by this system. Charge difference resulting from phosphorylation could be expected to increase mobility in SDS gels. The effects on migration of methylation

TABLE I

Migration (R_f values) of adult and embryonic H1 histones

H1 Histone	n in sample	Mean R_F	S.D.
H1m	2	875	±3.54
H1g	1	856	—
Adult I	5	855	±3.42
Adult II	5	895	±2.19
Sperm I	2	817	±2.83
Sperm II	2	828	±0.00

or acetylation are minimal in SDS gels. Because of their slower migration, the bands resolved in sperm and testis (Sperm H1-I and II) are unlikely to be the result of phosphorylation of H1g or some other H1 co-migrating with H1g. Also, phosphorylation of H1 histone is closely related to cell division, which is absent in sperm. Therefore this possibility seems remote (Easton and Chalkley, 1972).

If the Adult H1-II and sperm H1's do represent valid H1 subtypes, it is evident that histone genes coding for these variants are under control at either the transcription, processing, or possibly the translational level during embryogenesis and spermatogenesis. Testis shares the H1 histones found in somatic tissues, in addition to having the subtypes found in sperm. The presence of a more slowly migrating H1 band in sperm was initially reported by Easton and Chalkley (1972), using acid-urea gels to resolve histones of *Arbacia punctulata.* Those authors concluded that perhaps the sperm H1 subtype is somehow related to the greater degree of compaction of sperm chromatin. H1 histones have been implicated in the superstructure of chromatin by virtue of their position as bridges between nucleosomes (Newrock *et al.,* 1977).

The application of ion-exchange chromatography to the H1 histones of adult sea urchin tissues might well reveal additional tissue specific subtypes, reflecting the various cell phenotypes in specialized tissues.

Brandt *et al.* (1979) reported two H1 bands from the gut of *P. angulosus,* resolved on acid-urea gels. Since the system for resolving the proteins differs from the method described here, the results are not easily compared.

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AGONISTIC BEHAVIOR IN THE INTERTIDAL SEA ANEMONE *ANTHOPLEURA XANTHOGRAMMICA*

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ABSTRACT

The large intertidal sea anemone *Anthopleura xanthogrammica* (Brandt) forms aggregations of genetically distinct individuals at exposed sites along the west coast of North America. Individuals are often in close tentacle contact and remain that way for several years. However, this species displays the same agonistic behavior, inflation, and application of acrorhagi, as does its smaller clone-forming congener *Anthopleura elegantissima.* The behavior is frequent and more common among large individuals than small ones. Field transplants were used to show that non-neighbor anemones elicit the full acrorhagial response but that neighbors in close contact fail to behave agonistically towards each other. Microscopic examination of tissue samples revealed that conflicts between opposite sex anemones were as common as those between same sex anemones and that neighbors found in close contact were also as likely to be the same sex as the opposite.

Field anemone removal experiments in crowded pools and channels were used to compare subsequent movement of anemones released from crowding to movement of anemones never crowded and of those continuously crowded. Newly released anemones do move away from their neighbors but generally do not move out of tentacle contact. These results support the hypothesis that the application of acrorhagi serves more of a communication function than one of severe intraspecific competition. An anemone that has been attacked receives information on the size and position of its neighbor and can then move or not depending on available space. If movement is not possible, the agonistic behavior of its neighbor probably decreases or disappears altogether as a result of habituation.

INTRODUCTION

The existence of agonistic behavior among sea anemones was one of the most surprising additions to our knowledge of coelenterate behavior. Certain anemones use acrorhagi, vesicular extrusions at the margin of the column, to damage anemones of other species and even anemones of different genotypes within the same species (Abel, 1954; Bonnin, 1964; Francis, 1973a, b, 1976; Bigger, 1976, 1980; Brace and Pavey, 1978; Sebens and Paine, 1978; Ottaway, 1978; Williams, 1978, 1980; Brace *et al.* 1979; Brace, 1981; Ayre, 1982; Sebens, 1982b). Other anemones employ elongate (catch) tentacles as aggressive organs (Williams, 1975; Purcell, 1977; Kaplan, 1983) or use their feeding tentacles to damage other anemones and corals (Sebens, 1976). Similar sweeper tentacles also occur in another anthozoan group, the scleractinian corals (den Hartog, 1977; Richardson *et al.*, 1979; Wellington 1980) and are used agonistically as a mechanism distinct from the extrusion of mesenterial filaments and extracoelenteric digestion of neighboring corals (Lang, 1971, 1973; Connell 1976;

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Wellington, 1980). Anemones are not known to extrude mesenterial filaments as an agonistic behavior. If anemones evolved from coral ancestors as suggested by Hand (1966), it appears that the extracoelenteric digestion behavior was lost and that acrorhagi evolved as new organs associated with agonistic behavior. Acrorhagus-like organs are also found in at least one corallimorpharian, a group morphologically intermediate between corals and anemones (den Hartog, 1977).

All anemone species bearing acrorhagi use them agonistically (Bigger, 1980), both interspecifically and intraspecifically, although anemones that reproduce asexually do not attack members of the same clone (Francis, 1973b, 1976, Bigger, 1976, 1980; Ayre, 1982; Sebens, 1982b). A possible exception to this observation was the large intertidal anemone *Anthopleura xanthogrammica* from the Pacific coast of North America. Although its acrorhagi are well developed, agonistic behavior was either never observed (Francis, 1973b) or observed only once in many hours of observation (Lindberg, 1976; Bigger, 1980; Sebens, 1983; Koehl, pers. comm.). Francis (1973b) suggested that acrorhagi were vestigial in this species, which forms dense aggregations of unrelated individuals in close contact (Batchelder and Gonor, 1981; Sebens, 1981a, b, d, 1982a, 1983). These anemones do not maintain a wide and regular spacing between individuals (as in *Actinia equina*, Brace *et al.*, 1979; *Phymactis clematis*, Sebens and Paine, 1978; Brace, 1981, or the solitary form of *Anthopleura elegantissima*, Francis, 1979; Sebens, 1983). *A. xanthogrammica* does not reproduce asexually; it reproduces sexually by broadcast spawning followed by external fertilization and a lengthy planktonic development (Seibert, 1974). The present study was initiated to determine the importance of acrorhagi to *A. xanthogrammica* under field conditions.

Recent studies have shown that another anemone, *Metridium senile*, forms mixed-genotype aggregations in the field and habituates to non-clonemate anemones, reducing the frequency of agonistic behavior with time (Purcell and Kitting, 1982). Kaplan (1983) suggested that agonistic behavior does not occur between *Metridium* clones of opposite sex and that individuals of opposite sex will comingle on artificial substrata in the field. The possibilities that *A. xanthogrammica* in contact in the field have 'habituated' to each other, or that opposite sex contacts are more frequent than same sex contacts, were also investigated.

MATERIALS AND METHODS

Field studies were begun during September 1982 and continued during September 1983 at Tatoosh Island, Washington (48°27′N; 122°57′W), a very exposed open coast location at the northwest tip of the Olympic Peninsula. Laboratory observations on several hundred *Anthopleura xanthogrammica* and extensive field observations at low tide (1973–1977) failed to show agonistic behavior between individuals in close contact although one aggressive encounter by an *Anthopleura elegantissima* in an aquarium was followed by acrorhagi inflation and application by a large *A. xanthogrammica* (10 cm diameter). A single instance of agonistic behavior was observed in the field during a SCUBA dive at Tatoosh Island (1–2 m depth) in which two adjacent individuals (12–14 cm diameter) were observed with acrorhagi inflated. One raised the acrorhagi and brought them down onto the other individual. Given these observations and the two single observations reported in the literature (Lindberg, 1976; Bigger, 1980) it appeared that anemones kept in the laboratory were not behaving normally and that extensive field observations, when anemones were submerged, might be required to investigate the behavior. *A. xanthogrammica* lives in low intertidal habitats with extreme wave action and specimens in flowing sea water aquaria rarely expand to the degree seen in the field; they will often refuse suitable food and are thus not behaving normally. Anemones observed in tide pools and rock crevices at low tide are often wholly or partly contracted. Those that are expanded are in quiescent water and their tentacles are thus not being brought into contact with neighbors even a few centimeters away.

During September 1982 observations of anemones were made just after mid-tide for 2–4 h by snorkeling (at areas 3, 7, 9, and M-3 in Fig. 3, Sebens, 1983). This area receives moderate wave action but is just protected enough to allow snorkeling (diving); it includes approximately 300 anemones. Anemones are generally less crowded here than in more exposed pools and crevices but some pools can be found where most of the available space is occupied and anemones are in close contact. Even where sparse, a few anemones can be found in pairs or triplets with obvious contact between tentacles. Inflation and application of acrorhagi were observed several times during this period. Positions of the anemones were mapped and the basal diameters of each anemone involved in an interaction were measured and recorded on the next low tide.

Experimental encounters were also effected during this period. Anemones (7–12 cm diameter) were carefully removed from the rock and transplanted into contact with one or two other anemones at locations > 2 m from their initial site of attachment. The transplanted anemones were enclosed in wide mesh (chicken-wire) enclosures sealed at the bottom so that each anemone was tightly enclosed but the tentacles and parts of the column protruded through the wide mesh. These enclosures were then attached to masonry nails driven into cracks or holes in the rock using nylon cable ties. This method kept the transplanted anemone in contact with resident (subject) anemones and prevented movement of the enclosure during wave surge. Eighteen transplants were set up during morning low tides and monitored by snorkeling as the tide rose in the afternoon. Yellow plastic flagging tape was attached to one of the nails to facilitate recognition of the experiments under water. Basal diameters of all individuals in the transplants were measured, and thus the sizes of all anemones that either did or did not inflate the acrorhagi were known. Ten additional transplants were effected in which a large (3 cm) *A. elegantissima* was moved into contact with one or two *A. xanthogrammica.*

The same protocol was used during September 1983. Twenty-two transplants of individuals separated by 2 m or more were effected. In addition, 27 anemones which were originally in close contact with at least one other (≤1 cm between basal disc edges) were each transplanted to the opposite side of their original neighbors. Given their slow rates of movement, anemones in such close contact must have been in contact for at least several days and probably much longer. Therefore, the potential for habituation existed and, if this had occurred, such pairs should have shown little or no agonistic behavior toward each other.

Anthopleura xanthogrammica has maximum gonad development and is fully mature in September and spawns in October or slightly later (Sebens, 1981d). All anemones used in experiments or observed in agonistic encounters were sampled for later sex determination. Another 24 pairs in close tentacle contact were also sampled for sex determination. Wedges of the anemones were cut out with a razor blade (20–50% of the anemone), placed into numbered plastic bags, and preserved with 7.5% formalin in sea water. In October 1983 all anemone samples were hand-sectioned at several levels of the column and the 1–3 mm thick sections were examined at 20–40× under a dissecting microscope. When gonads were found, pieces were removed, pressed gently below a coverslip, and examined at 100× with a compound microscope. Oocytes, mature ova, and testis tubules were evident in these preparations.

Anemone movement was investigated by mapping anemones in photographs taken during 1974–1976. Clearings had been made in tide pools and channels to follow the rate of immigration of new anemones (Sebens, 1981a, b). For the present study, all anemones bordering the removal areas were mapped from projections of the photographs. The positions of anemones were re-mapped five times during the following 12–15 months. Movement was slow enought that individuals could easily be identified based on their relative size and orientation. In a few cases a new small individual appeared and previously one tens of centimeters away had disappeared. If no other major rearrangement occurred, it was assumed that this represented the same individual. The shortest possible path of each anemone was diagrammed for each interval and the total linear movement was summed at 3–4 and 12–15 months. This represents the least possible movement between sampling visits that could produce the new pattern.

Bordering individuals were separated into groups: those surrounded by other anemones and/or pool and channel walls (crowded), those released from crowding on one side by the experimental removal of their neighbors, and those that were initially uncrowded. An adjacent unmanipulated area (control M-3, Sebens, 1983) was also mapped by the same technique to measure the movement of initially uncrowded anemones. Any anemone in contact with one or no others was considered uncrowded. Measurements on anemones experimentally released from crowding should indicate whether the presence of neighbors causes anemones to move away if there is suitable space available. The adjacent space is known to be suitable because of its prior occupation by crowded adult anemones.

RESULTS

Agonistic behavior in the field

Thirty-four natural agonistic encounters were observed during this study. An agonistic encounter between two *Anthopleura xanthogrammica* is identical in sequence and postures to that described for *A. elegantissima* (Francis, 1973b). After initial contact by the tentacles or column of a neighboring individual, the first anemone to initiate the behavior (termed "aggressor") pulls the tentacles back and up from the region of contact, bends away, then inflates the acrorhagi which are translucent green with pale green to white tips. The acrorhagi are held almost perpendicular or at least at an angle of 45° to the substratum at this point. The acrorhagi are then brought down on the other anemone in a sweeping motion by the contraction of the column on the side with the inflated acrorhagi. The tentacles are usually bent away from the acrorhagi or even partially deflated (Fig. 1). The sweep continues downward and causes the acrorhagi to contact the tentacles and then most of the column of the other (termed "attacked") anemone, leaving patches of light-colored dehiscent ectoderm from the acrorhagi (peeling) on the dark column of the attacked anemone. Ectoderm also adheres to the tentacles of the attacked anemone but is less readily seen because of the lack of color contrast and the fact that the tentacles are rapidly contracted. The downward sweep stops at the base of the attacked anemone and the aggressor's acrorhagi are often deflated before it again assumes its erect posture. After this sequence the aggressor either returns to its normal expanded posture with no acrorhagi visible or, in fewer cases, contracts and retracts the tentacles. The entire sequence from inflation of the acrorhagi to the return to normal posture lasted nine minutes in one timed episode, but was sometimes even more rapid. The attacked anemone almost invariably contracts, retracts the tentacles, and remains in this posture for minutes to hours. Only in two cases did the attacked anemone inflate acrorhagi during the observation and thus continue the agonistic encounter.

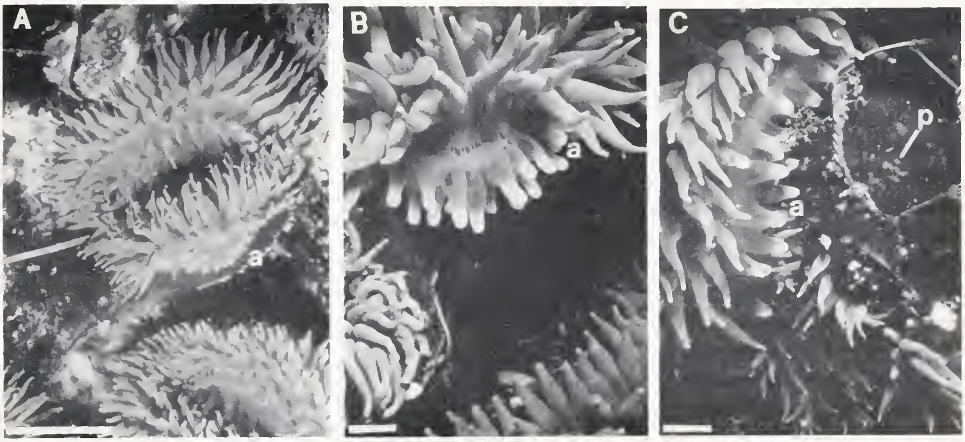

FIGURE 1. A. *Anthopleura xanthogrammica* agonistic encounter, Tatoosh Island, Washington. The individual at the top has its acrorhagi (a) inflated on the side facing its neighbor. Scale bar = 5 cm. B. Acrorhagi of a subject anemone prior to contact with an experimentally transplanted anemone in a wire enclosure. Scale bar = 1 cm. C. Acrorhagi tip ectoderm (light patches (p)) adhering to the column of a transplanted anemone (peeling). Scale bar = 1 cm.

The short duration of the behavior and the color similarity between tentacles and acrorhagi make this behavior easy to miss even during many hours of field observation. Also, it appears that water movement is the most common means by which anemones come into contact, as noted by Francis (1973b) for *Anthopleura elegantissima.* Anemones in tide pools or aquaria are thus much less likely to show the behavior. I have seen only two agonistic encounters in tidepools and one of these occurred as I stirred the water by reaching into the pool to measure anemones. My impression from snorkeling observations is that the behavior occurs most frequently during wave action. It takes only 10–15 minutes for the anemones to fully expand when the tide comes in. When the water is a half meter or less deep even small waves knock the anemones into each other. Of all the agonistic behavior observed, most was between anemones 4–6 cm apart (10 pairs) although there were cases where the anemones were 0–3 cm apart (6 pairs). The mean distance between fighting anemones was 3.0 cm ± 2.3 cm S.D. ($n = 16$).

The entire study area contained 283 *Anthopleura xanthogrammica* of which 168 were in close contact (≤ 4 cm between bases) with at least one other individual of the same species. During the 1982 study twelve incidents of intraspecific agonistic behavior were observed (out of 98 anemones in potential contact) and 6 incidents of such behavior between *A. xanthogrammica* and *A. elegantissima.* During the 1983 study period 18 intraspecific agonistic incidents were observed in the same area (out of 77 anemones in potential contact) and 3 instances of agonistic behavior with *A. elegantissima.* Anemones were observed for a total of 16 out of 84 hours during which the anemones were submerged, which was 19% of the total time when agonistic behavior could have been observed. The average frequency of intraspecific agonistic behavior in 1982 was thus .25 incidents per adult anemone in contact with others per day and .26 incidents in 1983. Accordingly, an anemone would experience one agonistic encounter every four days on average.

However, not all anemones are equally likely to participate in agonistic interactions (Fig. 2). Anemones with ≤ 7 cm basal diameters displayed agonistic behavior in only three cases, out of 36 anemones (8%) during the study. Anemones with ≤ 6.5 cm

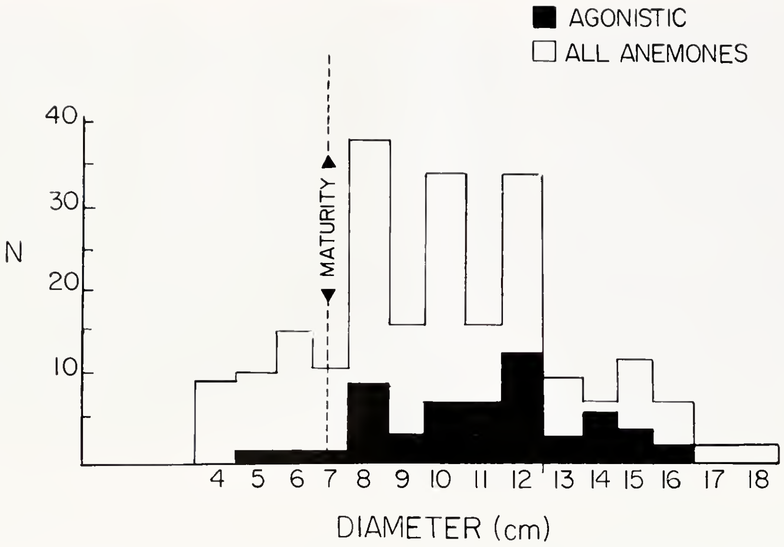

FIGURE 2. Sizes of *Anthopleura xanthogrammica* that displayed agonistic behavior (acrorhagal inflation) compared to the size distribution of all anemones mapped during the study.

basal diameters were found to be immature (juveniles) in this and an earlier study (Sebens, 1981d) while most individuals above that size had obvious male or female gonads. Anemones with 7.5–10 cm basal diameters displayed 19 agonistic behaviors out of 88 anemones observed (22%) and anemones > 10 cm displayed 35 such encounters out of 90 anemones (38%). Clearly juveniles are unlikely to participate in agonistic encounters either as aggressor or as defender, while adults do so frequently. A chi-squared test was performed on juvenile and adult categories assuming as the null hypothesis that agonistic behavior would be observed equally in both groups. The hypothesis must be rejected ($\chi^2 = 13.7$, $P < 0.005$) and the frequency of such behavior is thus significantly lower among juveniles. There was no significant difference between large (>10 cm) and small (≤10 cm) adults. One hypothesis for the low incidence of agonistic behavior in juveniles results from their small size and perimeter: the probability of contacting other anemones would be lower among smaller individuals compared to larger individuals. However, small anemones move more frequently (Sebens, 1981b, this study) and could experience even more contacts per unit time than do the adults.

For all natural agonistic interactions, the size of the aggressor was plotted against that of the anemone that had been attacked (Fig. 3). In some cases the initiation of aggression could have been missed and only the response seen. However, most observation periods began soon after the tide covered the anemones and continued for several hours. In only two cases was there a responding inflation and application of acrorhagi by the attacked anemone. Others may have done so after the period of observation, meaning a lag time of up to several hours between stimulus and response. For 21 of 28 anemones in Figure 3 the aggressor was larger than the attacked anemone; for 6 of 32 they were equal; and for 2 of the 28 the aggressor was smaller. A chi-squared test on the data in Figure 3, assuming equal frequencies of aggression by the larger and by the smaller member of a pair as the null hypothesis, shows that the opposite was true ($\chi^2 = 12.9$, $P < 0.005$); the aggressor was significantly larger. In

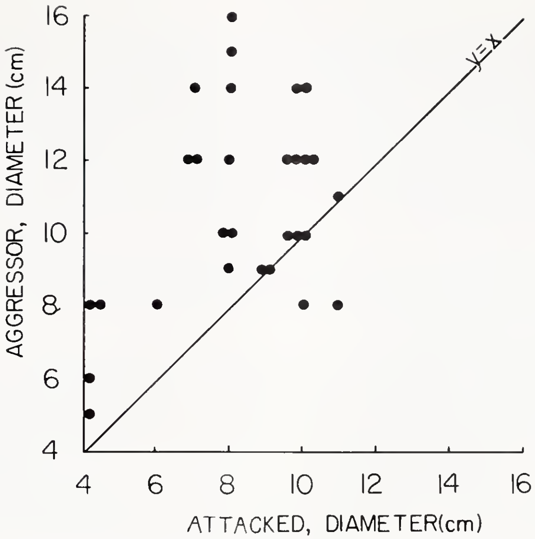

FIGURE 3. Sizes of anemones in pairwise natural agonistic encounters. The first or only anemone seen to inflate the acrorhagi is termed the "aggressor."

general, larger anemones appear to be both more aggressive and more likely to attack individuals smaller than themselves.

For the adult anemones, the frequency of agonistic behavior was 30% of all anemones observed in potential contact (in 19% of the time submerged, 1983). From these data, the estimate of the frequency of agonistic behavior becomes .34 incidents per anemone per day in the study area. This estimate establishes that agonistic behavior in *A. xanthogrammica* is very common in natural populations.

During the same study period nine incidents of agonistic behavior were observed between *Anthopleura xanthogrammica* and *A. elegantissima.* In six of those incidents only *A. elegantissima* inflated the acrorhagi and in two incidents only *A. xanthogrammica* did so. In one case an *A. elegantissima* inflated the acrorhagi after being attacked by an *A. xanthogrammica.* In no case was there a response by *A. xanthogrammica* to a previous aggression by *A. elegantissima.* The only other anemone occurring with *A. xanthogrammica* in this study was *Tealia coriacea* (eight individuals observed), but no agonistic encounters were observed between *A. xanthogrammica* and this species. In 1982, 11 *A. elegantissima* transplants were placed in contact with *A. xanthogrammica;* of these only 2 (18%) elicited acrorhagi inflation by *A. xanthogrammica.* However, the small size of *A. elegantissima* may have precluded contact in some cases.

On the most exposed points of Tatoosh Island, *Anthopleura xanthogrammica* often carpets the floors of tidepools and channels, sometimes so densely that another adult anemone could not be added easily (Sebens, 1981b, 1983). There were several smaller pools that had a similar dense packing of anemones in the area used for this study. Even where space was obviously available and anemones were not crowded it was easy to find pairs or greater numbers of anemones clearly in contact with each other. *A. xanthogrammica* does not reproduce asexually, and its planula larvae develop in the plankton and spend at least several weeks before settling (Siebert, 1974). It is, therefore, extremely unlikely that neighboring anemones are any more closely related than those not in contact. Since the anemones fight frequently yet tolerate close contact in many cases, there is potential for the type of 'habituation' that occurs in *Metridium senile* (Purcell and Kitting, 1982). After 3–6 days of agonistic behavior, using catch-tentacles, *Metridium* essentially stops agonistic behavior. If this had occurred between neighboring *A. xanthogrammica* it should be possible to remove one of the neighbors and transplant it to the opposite side without the remaining anemone acting aggressively towards it. On the other hand, anemones transplanted from a distance (≥2 m) away should elicit agonistic behavior from one or both members of such pairs. It was not possible to test for habituation in the laboratory because the behavior of *A. xanthogrammica* is never completely normal in aquaria and the agonistic behavior cannot be reliably induced. Given more time in the field it would be possible to make permanent transplants of non-neighbor anemones to see if agonistic behavior towards them declines with time.

During the 1982 study period, 18 non-neighbor transplants were carried out. Of these, six subject anemones behaved aggressively towards the transplant (Fig. 4). Transplanted anemones had much of their columns and usually some of their tentacles protruding through the wide mesh as they expanded in the confined space. During the 1983 period, six of 32 subject anemones attacked the transplants, for a total of 24% showing agonistic responses during the observation period (in 19% of total submerged time). This translates to a maximum of 1.3 such behaviors per subject anemone per day, much higher than the .34 interactions in the population at large. Twenty seven transplants of anemones originally in close contact were carried out during the 1983 study period. Only one (3.7%) of the subject anemones showed agonistic behavior (Fig. 4), which would give a maximum estimate of .20 agonistic encounters per neighboring anemone per day. A chi-squared test was performed, assuming equal frequency of agonistic behavior in each group as the null hypothesis. The hypothesis

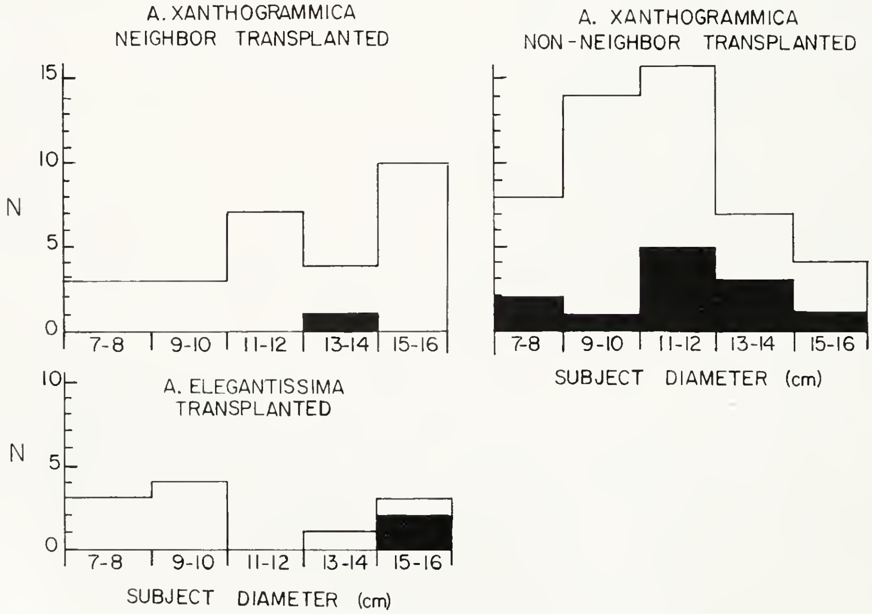

FIGURE 4. Sizes of anemones that did or did not display agonistic behavior (acrorhagal inflation) during transplant experiments. Light bars include all subject anemones, dark bars only those that showed agonistic behavior.

was rejected ($\chi^2 = 4.25$, $P < 0.05$); there was significantly more such behavior in the transplants of originally distant anemones. Clearly the response to contact with non-neighbors is very strong, but that for neighbors is almost nonexistent. One explanation for this result is that neighbors have habituated to each other and that the agonistic behavior occurs infrequently if at all. Another possible explanation is that neighbors have some special characteristics (probably not relatedness), such as the sexual sorting out suggested by Kaplan (1983) for *Metridium senile* in which anemones fail to attack those of the opposite sex.

The analysis of gonad sections from the 1983 study is summarized in Table I. Anemones were classed as either male or female adults, juveniles (≤7 cm), or without gonad ('not determined,' >7 cm). Of the 15 natural agonistic encounters observed and sampled, 4 were between obvious adult male and female anemones, 4 were between anemones of the same sex, 2 involved adults and juveniles and 5 involved anemones of undetermined sex. Of 19 naturally occurring neighboring anemones (≤2 cm between bases) that did not fight, 4 were male-female pairs, 5 were of the same sex, 3 were adults with juveniles, and 7 were between anemones of undetermined sex. There is thus no sorting out into male-female pairs nor is agonistic behavior limited to encounters with anemones of the same sex. Among the transplanted and subject anemones the same result held; neighbors included both same sex (7) and opposite sex (5) pairs. Non-neighbor transplants that fought included opposite (2) as well as same sex (2) pairs and those that did not fight also included opposite (9) and same sex (13) pairs. Based on these findings, sex does not appear to influence agonistic behavior nor does the reproductive state of the adults; adult individuals without gonads engaged in several agonistic encounters as did those with gonads.

Movement in response to neighbors

Anemones bordering experimental anemone removal areas were classed as adults or juveniles by their size and were also categorized as to their initial situation: (1) uncrowded, (2) crowded, and (3) crowded then released from crowding, on one side, by the experimental removal of neighbors. The removal experiments were originally

TABLE I

Sex categories of anemone pairs used in experiments, found in close contact, or displaying agonistic behavior (136 anemones dissected and examined)

	Total Pairs	Male/ Male	Female/ Female	Male/ Female	Male/ Juv.	Female/ Juv.	Male/ Neut.	Female/ Neut.	Neut/ Neut.
Natural agonistic encounters	15	1	3	4	1	1	3	2	0
Pairs in close contact	19	4	1	4	1	2	1	5	1
Transplants: close contact	20 (1)	3	4	5	2	0	2 (1)	4	0
Transplants: originally distant	34 (6)	4 (1)	7 (1)	9 (2)	1	0	3	7 (1)	3 (1)
Total agonistic encounters	22	2	4	6	1	1	3	3	1

Numbers in parentheses in the transplant groups are those that did behave agonistically.
Juv. = juvenile, Neut. = unable to determine sex.

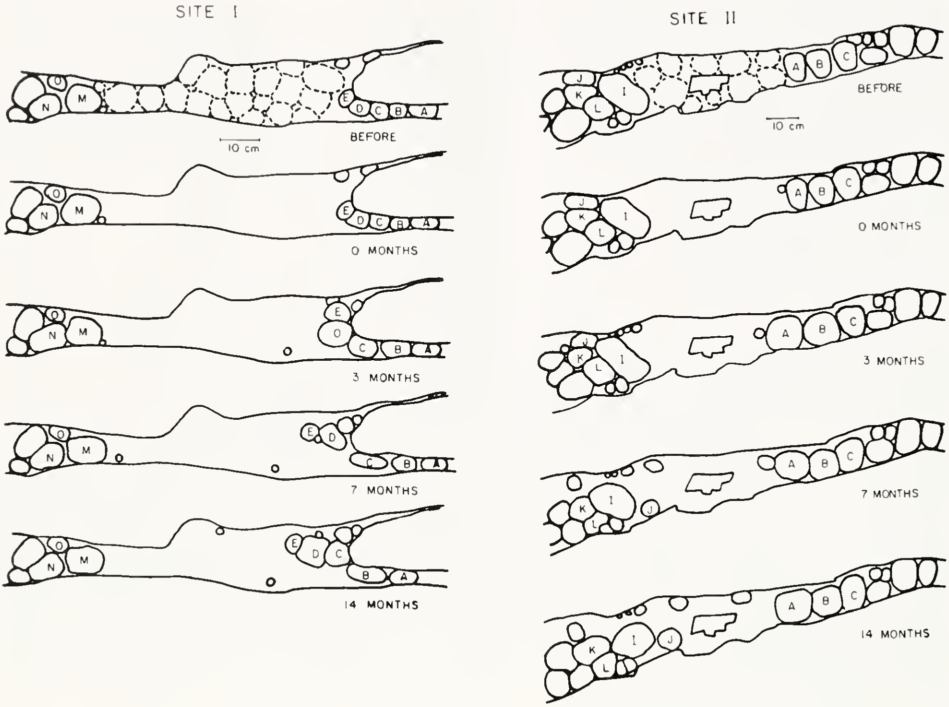

FIGURE 5. Two experimental anemone removal areas on Tatoosh Island. Dashed circles represent anemones removed from the channels, solid circles represent those that remained. Adult anemones close to the removal areas are identified by letters. Note that individuals A–E at site I moved substantially but each remained in contact with one or more neighbors. Only anemones A and J and a few juveniles moved measurably at site II.

conducted to measure the rate of immigration and the sizes of immigrating anemones (Sebens, 1981a, b), not to measure movement as a function of initial position. Only the removal areas within approximately 30 m or less of the 1982–1983 study areas were analyzed in this study.

Movement was greatest among both adult (≥7 cm diameter) and juvenile (<7 cm) anemones newly released from crowding (Figs. 5, 6). Solitary anemones and continuously crowded anemones moved much shorter distances, if at all. Analysis of Variance (ANOVA), followed by a Student-Newman-Keuls multiple comparisons test (SNK), was used to test for differences in total summed linear displacement after 3–4 months, and after 12–15 months (Sokal and Rohlf, 1968). After 3–4 months, the released adults had moved farther than either the crowded or solitary adults (ANOVA, $F_s = 24.8$, d.f. = 2,70, $P < 0.001$), but there was no difference between either of the latter groups (SNK). The same was true after 12–15 months ($F_s = 35.5$, d.f. = 2, 69, $P < 0.001$). Among the juveniles, the released group differed only from the solitary ones after 3–4 months ($F_s = 2.7$, d.f. = 2,31, $P < 0.05$) and only from the crowded ones after 12–15 months ($F_s = 3.2$, d.f. = 2,31, $P < 0.05$). Juvenile movement was significantly greater than adult movement only after 12–15 months in the solitary group ($F_s = 5.9$, d.f. = 1,48, $P < 0.05$) and not among crowded or newly released anemones (Fig. 6).

These results suggest that adult movement is extremely slow, only tens of centimeters in a year, and that juvenile movement is only about twice as rapid. However,

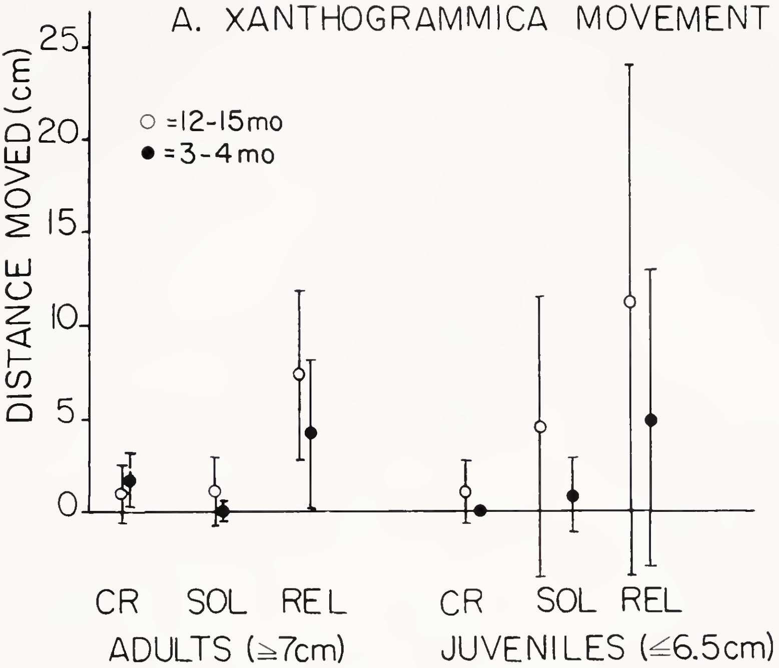

FIGURE 6. Distances moved by all anemones bordering the experimental anemone removal areas. CR = continuously crowded anemones, SOL = solitary anemones, REL = anemones released from crowding by the experimental removal of their neighbors.

far more juveniles than adults moved into all experimental removal areas during 1973–1977 (Sebens, 1981a, b, 1982a, b). Many of these juveniles came from >.5 m away because they did not appear in the map from the previous sampling period. The rates of juvenile movement in this study omit all juveniles not present at initiation and may thus greatly underestimate juvenile mobility.

It is clear that continuously crowded adults and adults with abundant available space adjacent to them hardly moved at all (Fig. 7). Anemones crowded before the experiment, then given free space, moved into it but did not separate themselves from neighboring anemones by any great distance, usually remaining close enough for tentacle contact. The mean distance between nearest neighbor basal discs of newly released anemones after 12–15 months was 0.9 cm ± 1.9 cm S.D. (n = 20) as opposed to 0.2 cm ± 0.5 cm S.D. (n = 20) before removal. These means are not significantly different. From this experiment it appears that *A. xanthogrammica* will move away from conspecifics if space is available but that they do not maintain a wide spacing between neighbors as do other large non-cloning anemones (Solitary *Anthopleura elegantissima, Phymactis clematis, Phymanthea pluvia*) which behave agonistically (Sebens and Paine, 1978; Francis, 1979; Brace, 1981) and maintain at least one body diameter between neighbors.

DISCUSSION

The large intertidal anemone *Anthopleura xanthogrammica* displays an acrorhagal agonistic behavior almost identical to that of its sympatric congener *A. elegantissima* (Francis, 1973b, 1976). The behavior increases in frequency with anemone size in

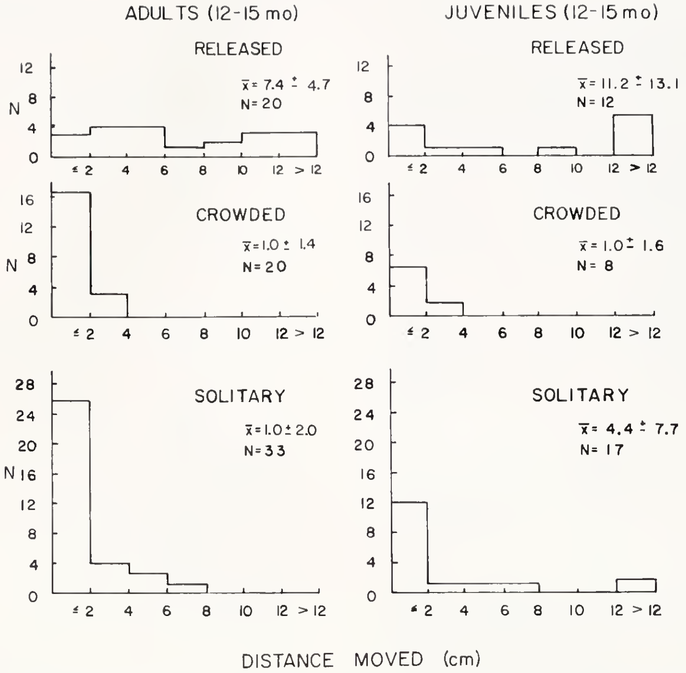

FIGURE 7. Distribution of distances moved by juvenile and adult anemones bordering the experimental anemone removal areas. "Crowded" anemones were those continuously crowded, both before and after the removal. "Solitary" anemones were in contact with one or no other anemones before and after the removal. "Released" anemones were released from crowding by the experimental removal of their neighbors. x̄ ± S.D. are given as cm moved.

the field and, among large anemones, may occur as frequently as once per day. Individuals found in contact, and which have probably been in contact for long time periods, are far less likely to behave agonistically toward each other than are widely separated anemones. This tolerance for contact with genetically dissimilar adults led Francis (1973b) to postulate that the acrorhagi were possibly vestigial in this species. Bigger (1980) and Lindberg (1976) reported single cases of acrorhagal inflation and agonistic behavior in tidepool and aquarium situations respectively. Prior to this study I saw only one intraspecific agonistic episode in the field and one between *A. xanthogrammica* and *A. elegantissima* in a flowing sea water aquarium. The present field observations and experiments suggest that agonistic behavior is frequent and important in natural populations of *A. xanthogrammica. Anthopleura elegantissima* recognizes genetically identical clonemates and confines agonistic behavior to encounters with non-clonemates and to other anemone species (Francis, 1973b, 1976; Sebens, 1982b). Recognition of non-clonemates is based on intact tissue surfaces (Lubbock, 1980) and may be related to 'immunoincompatibility' as suggested for corals (Hildemann *et al.,* 1975, 1977, 1979; Bigger and Runyan, 1979; Bigger, 1980). However, surface mucus will elicit nematocyst discharge in interspecific encounters of several anemone species (Lubbock, 1979; Ertman and Davenport, 1981) and may be the general basis for recognition of other anemone species, sometimes followed by initiation of agonistic behavior. *A. xanthogrammica* does not reproduce asexually (Hand, 1955; Sebens, 1983) and reproduces sexually by spawning gametes into the sea water where fertilization and subsequent development occur (Siebert, 1974). Planula larvae require several weeks to fully develop and possibly longer before they are ready to settle. Individual anemones at any one site are thus unlikely to be closely related, and relatedness cannot be a factor in tolerance of contact by adult conspecifics, unless there are 'incompatibility groups' based on shared alleles (as in corals, Hildemann *et al.*, 1977). It is more likely that *A. xanthogrammica* can recognize all or most other conspecific individuals and all individuals of other species as 'not-self' as do individual *A. elegantissima* (Francis, 1973b, 1976, 1979), *Actinia tenebrosa* (Ayre, 1982), and *Anthopleura krebsi* (Bigger, 1976, 1980) from distinct clones.

Tolerance of contact with conspecific adults is not sex dependant (as suggested for *M. senile,* Kaplan, 1983) and most likely results from habituation (as in *Metridium senile,* Purcell and Kitting, 1982). Same-sex pairs in contact were as common as those of opposite sex and agonistic behavior occurred just as frequently between anemones of the same sex as between those of opposite sex. This tolerance of contact, and the infrequent agonistic behavior during periods of still water, or in aquaria, are probably the factors that prevented previous recognition of the importance of agonistic behavior in this species.

Pools and channels at exposed sites can contain hundreds of individuals per square meter, most of which are in contact with one to three others (Sebens, 1983). Areas with lower overall density also contain many pairs or greater numbers of individuals in contact. A wide inter-individual spacing obviously does not occur as a result of agonistic behavior. Other large intertidal anemones that do not reproduce asexually (*Phymactis clematis* in Chile, Sebens and Paine, 1978; Brace, 1981; *Phymanthea pluvia* in Chile, Sebens and Paine, 1978; *Anthopleura elegantissima* solitary form, Francis, 1979) usually maintain spaces of one or more individual diameters between individuals by bending the column far over and reaching out with the tentacles and then with the acrorhagi. *Actinia equina* (Brace *et al.,* 1979), *A. tenebrosa* (Ottaway, 1978; Ayre, 1982), *Bunodactis cavernata* (Bigger, 1980), and *Anemonia sargassensis* (Bigger, 1980) are also reported to maintain a distance between individuals as a result of agonistic behavior.

Crowded adult *Anthopleura xanthogrammica* that were experimentally provided with suitable space on one side moved significantly more than did either continuously crowded or uncrowded anemones in this study. Although they did not establish a wide spacing between individuals, they did move away from the crowded areas of pools and channels; they also increased the distance between themselves and their neighbors, although usually not by enough to prevent frequent tentacle contact. Therefore, these anemones do move in response to crowding, although slowly. Agonistic behavior may thus be a form of information transfer, communicating the position of neighbors to any one anemone. If suitable space is available, the anemone bordering that space could then move into it and away from its neighbor.

Excessive crowding probably causes the anemones' tentacle crowns to interfere with each other. This could reduce the probability of prey capture [dislodged mussels, barnacles, and other invertebrates (Sebens, 1981c)] by reducing the total surface area of the tentacle crown; shading by other anemones could also reduce the photosynthetic rate of symbiotic algae. In fact, crowded anemones grew less rapidly than did those experimentally released from crowding in a previous study (Sebens, 1983). This effect was most obvious in the intermediate (6–10 cm diameter) size range. Anemones ≤6.5 cm diameter were highly mobile; they appeared and disappeared from study areas frequently and accounted for most of the recruitment into cleared areas (Sebens, 1981a, b, 1983). Reduced crowding, even though it may still involve tentacle contact, could thus be advantageous to all anemones concerned.

The extremely wave-exposed habitats occupied by *Anthopleura xanthogrammica* may impose a constraint on wide spacing. Koehl (1977) has noted that the relatively even surface formed by closely adjacent tentacle crowns of this species could reduce the shear forces tending to deform or detach the anemones. Instead of impinging on the side of each anemone, the rapidly flowing water during wave surge goes over their oral discs. In fact, solitary *A. xanthogrammica* are found more commonly in habitats with less exposure and in deep crevices at the exposed sites. Koehl (1977) also found that this species changes its shape, increasing the height to diameter ratio from the most exposed areas to those with less wave action. This suggests that forces imposed by water motion are indeed important. Biomechanical properties would thus select against a behavior that provides a very wide spacing away from all neighbors.

There are two other possible functions for intraspecific agonistic behavior in *A. xanthogrammica.* The first is competition for space within crowded pools and channels. The largest anemones often inhabit the centers of pools and channels and the smallest ones are limited to the sloping sides or are found between the large adults, overshadowed by their tentacle crowns (Sebens, 1981b, 1983). The small movements of the large anemones over months to years may represent a 'jockeying for position' within the pool. Even if most space is taken, there are still better and worse places to be. The central horizontal floor of pools and channels may well receive the most dislodged prey during periods of heavy wave action, and the least shading by surrounding rock, which would reduce photosynthesis by the symbiotic algae. The second possible function is defense of space from potential immigrants. Small anemones migrate into pools and channels at rates of several individuals per square meter per year (Sebens, 1981a, b). Without agonistic behavior by the residents, such anemones might crowd the area to the maximum. As it is, they are given information that parts of the area are already crowded and are thus encouraged to leave.

Anthopleura xanthogrammica also behaves aggressively towards *A. elegantissima* and may do so towards other species as well. At the sites examined, intraspecific contact was by far the most common but close proximity of *A. xanthogrammica* and *A. elegantissima* was probably the next most common situation. *A. elegantissima* individuals are generally ≤3 cm diameter at Tatoosh Island but can be very large at other sites (to 6 cm diameter) and even larger individuals of the solitary form are common in California (Francis, 1979). Therefore, agonistic behavior between these two species is probably common and may be an important mechanism of interspecific competition for space.

The influence of anemone size on agonistic behavior has been demonstrated for *Actinia equina* (Brace and Pavey, 1978; Brace *et al.,* 1979) and for *Phymactis clematis* (Brace, 1981). Large anemones have a lower threshold (less time from stimulus to response) for initiation of agonistic behavior and they tend to win contests with smaller individuals. However, *Actinia tenebrosa* does not fit this pattern; large anemones often lose to smaller ones (Ayre, 1982) and it appears that the resident anemone has a great advantage, winning most contests independent of size (Ottaway, 1978). Size is also important for *Anthopleura xanthogrammica* where larger individuals are more frequently the aggressors. The final outcome of each encounter is unknown because the anemones never moved perceptibly in the period available for study, but the attacked anemone often remained closed for hours. It is therefore most likely that the largest anemones are the dominant competitors in this species as well. Brace (1981) has suggested that the decrease in time to initiate agonistic behavior (after initial contact) as a function of anemone size serves a specific function; it prevents small anemones from starting or continuing fights that they can't win. The rapidity with which an anemone begins aggression following initial contact of two individuals conveys information on the probable size of that individual. If it aggresses first, it is probably larger. This may explain why so few of the attacked anemones in this study fought back. Once the size information is conveyed, the attacked individual need not continue the bout. Instead it remains contracted, avoiding further damage, and may slowly move away.

The acrorhagal agonistic behavior of *Anthopleura xanthogrammica* may well be an important means of competition for space, as it seems to be in the clonal species *A. elegantissima* (Francis, 1973b, 1976). Extensive damage to an attacked anemone was observed only once in this study, suggesting that the agonistic behavior serves more frequently as communication rather than as absolute aggression or defense. This idea is supported by the species' ability to reduce or eliminate the agonistic behavior after prolonged contact. An anemone that stands its ground may eventually be accepted by its neighbors, but it is to the intruder's advantage to move away if suitable space is available, thus avoiding severe overcrowding.

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SPECIFICITY IN THE INDUCTION OF POLYSPERMY IN SEA URCHIN EGGS BY SOYBEAN TRYPSIN INHIBITOR

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ABSTRACT

Sea urchin egg cortical granules contain a soybean trypsin inhibitor (SBTI)-sensitive protease that is activated and secreted upon fertilization. Results obtained in several laboratories have implicated this activity in the cortical reaction, elevation of the fertilization envelope, and the establishment of the block to polyspermy. These conclusions are supported in part by studies using a variety of trypsin inhibitors. This interpretation has recently been challenged, however, and past results with soybean trypsin inhibitor (SBTI) and other agents were attributed to a non-specific protein effect (Dunham *et al.,* 1982). We have re-examined this question by studying the effects of native and inactivated SBTI on purified egg protease and the promotion of polyspermy. Protease was purified from unfertilized eggs by SBTI-affinity chromatography in the presence of benzamidine-HCl to minimize autodegradation. Inactivation of SBTI by acid or alkali treatment greatly diminished its ability to inhibit the egg protease or to cause polyspermy. We also observed a correlation between the relative potencies of five serine protease inhibitors and their ability to promote polyspermy. We conclude that induction of polyspermy by SBTI and other protease inhibitors is indeed due to inhibition of the egg protease.

INTRODUCTION

The prevention of polyspermy in sea urchins is a multi-faceted process that includes structural, electrical, and enzymatic components. The block is usually considered to occur in two phases; an early but transient one, and a later but permanent one. Several events have been implicated in the early phase, including a rapid, sodium-dependent depolarization of the egg plasma membrane (Jaffe, 1976; Schuel and Schuel, 1981), the release of hydrogen peroxide by fertilized eggs which inactivates sperm in the vicinity (Boldt *et al.,* 1981; Coburn *et al.,* 1981), and the possible production of arachidonic acid oxidation products (Moss *et al.,* 1983; Schuel *et al.,* 1984). The permanent block to polyspermy is mediated by the cortical granule secretion-mediated lifting of the fertilization envelope which provides a stable physical barrier to further sperm penetration (reviewed by Schuel, 1978, 1984).

Evidence linking proteolytic activity in sea urchin eggs to the prevention of polyspermy, particularly with respect to the lifting of the fertilization envelope, has emerged over the last thirty years (reviewed by: Runnstrom, 1966; Epel, 1978; Schuel, 1978, 1984). Protease activity was observed early on in both unfertilized and fertilized eggs (Lundblad, 1954). In addition, soybean trypsin inhibitor (SBTI) and other trypsin inhibitors were found to impair fertilization envelope elevation and to cause polyspermy (Hagstrom, 1956). Subsequent studies showed that a SBTI-sensitive protease is localized

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in the cortical granules of unfertilized eggs (Schuel *et al.*, 1973; Decker and Kinsey, 1983; Kopf *et al.*, 1983) and is secreted during the cortical reaction at fertilization or upon parthenogenetic activation (Vacquier *et al.*, 1972a, b, 1973; Carroll and Epel, 1975a, b; Fodor *et al.*, 1975; Carroll, 1976; Schuel *et al.*, 1976b; Carroll *et al.*, 1982). A serine protease has been isolated from unfertilized sea urchin eggs and fertilization product by SBTI-affinity chromatography and has been characterized biochemically (Fodor *et al.*, 1975; Alliegro and Schuel, 1983a).

The biological functions of protease(s) in fertilization have been studied by using SBTI and other specific enzymatic inhibitors, and by the direct application of purified preparations of egg protease to fertilization cultures. The protease-dependent processes which may be related to the prevention of polyspermy include: discharge of cortical granules and detachment of the vitelline envelope from the egg plasma membrane (Hagstrom, 1956; Lonning, 1967; Vacquier *et al.*, 1972a; Longo and Schuel, 1973; Longo *et al.*, 1974; Schuel *et al.*, 1976a, b); generation of H_2O_2 by fertilized eggs (Boldt *et al.*, 1981; Coburn *et al.*, 1981); and the inactivation of sperm binding sites on the vitelline layer (Vacquier *et al.*, 1973; Carroll and Epel, 1975a). Polyspermy arises in SBTI-treated eggs by refertilization at specific sites where the vitelline layer fails to detach from the egg plasma membrane (Longo and Schuel, 1973; Longo *et al.*, 1974; Schuel *et al.*, 1976b). Carroll and Epel (1975a) proposed the involvement of two distinct SBTI-sensitive proteases in the block to polyspermy: vitelline delaminase, which cleaves bonds between the vitelline envelope and plasma membrane; and sperm receptor hydrolase, which digests sperm receptors on the surface of the vitelline layer.

This entire line of investigation has recently been challenged (Dunham *et al.*, 1982). These authors attributed the induction of polyspermy by SBTI to a non-specific protein effect, rather than the specific inhibition of egg proteases. Their conclusion was based on the observation that other proteins (catalase, superoxide dismutase, bovine serum albumin, and gelatin) which are apparently unrelated biochemically to SBTI also cause polyspermy in *Arbacia* eggs. This interpretation casts doubt upon past results using protease inhibitors and other agents as probes to study the mechanisms involved in polyspermy inhibition.

We have re-examined this question by studying the effects of native and inactivated SBTI on the promotion of polyspermy. If SBTI causes polyspermy by a non-specific protein effect as proposed by Dunham *et al.* (1982), then its capacity to do so would not be dependent upon its ability to inhibit the purified egg protease. This hypothesis was tested by taking advantage of the observation that acid or alkali treatment of SBTI removes its ability to inhibit bovine pancreatic trypsin (Kunitz, 1947). In addition, we have compared the effects of several other serine protease inhibitors on inhibition of the egg protease and the induction of polyspermy. A preliminary account of this study has been reported previously (Alliegro and Schuel, 1983b).

MATERIALS AND METHODS

Handling of gametes

Arbacia punctulata were obtained from the Marine Resources Department at the Marine Biological Laboratory, Woods Hole, Massachusetts, and *Strongylocentrotus purpuratus* from Pacific Bio-marine, Venice, California. Gametes were shed by intracoelomic injection of 0.5 *M* KCl (Harvey, 1956). Eggs were rinsed 2–3 times in filtered sea water (FSW). Sperm were stored "dry" on ice until needed.

Polyspermy experiments

Polyspermy experiments with protease inhibitors were carried out with *A. punctulata* gametes. Thirty microliters of settled eggs were preincubated for two minutes in 1.0 ml of FSW containing the desired inhibitor and inseminated with 40 μl of a 2.4% v/v sperm suspension. Based on an estimate of 2×10^{10} sperm/ml in dry semen (Harvey, 1956), the final sperm concentration in these experiments was approximately 2×10^7/ml. Lifting of the fertilization envelope was the criterion for determining fertilization. Polyspermy was estimated by counting the percentage of multipolar divisions at first cleavage (Schuel *et al.,* 1973). The incidence of fertilization was at least 95% in all experiments. Eggs were incubated at ambient temperature (24°–26°C) with occasional agitation. One hundred eggs were scored in each culture, and each experiment was repeated three times.

Enzyme isolation and assay

Protease was isolated from extracts of unfertilized *S. purpuratus* eggs by SBTI-affinity chromatography according to Fodor *et al.* (1975). Benzamidine-HCl (5 m*M*) was included to minimize autodegradation of the enzyme (Baginski *et al.,* 1982). Protease activity was measured by continuously recording the change in absorbance at 253 nm using benzoyl-l-arginine ethyl ester (BAEE) as substrate (Schwert and Takenaka, 1955). Reactions were initiated by adding 50 μl of the affinity-purified preparation (approximately 160 units/ml) to a cuvette containing 0.5 m*M* BAEE in 0.2 *M* Tris buffer, pH 8, and 25 μl of the appropriate inhibitor. One unit of activity is defined as $\Delta \text{Abs}_{253\ \text{nm}}$ of .001/minute. The total reaction volume was 2.5 ml. Inhibitors used were: soybean trypsin inhibitors (SBTI), lima bean trypsin inhibitor, chicken ovomucoid, antipain, and leupeptin. Antipain and leupeptin were gifts of Dr. Walter Troll. Soybean trypsin inhibitor-agarose for affinity chromatography was obtained from Bethesda Research Labs, Maryland. All other reagents were purchased from Sigma Chemical Co., St. Louis, Missouri.

Inactivation of SBTI

SBTI was inactivated according to Kunitz (1947). Briefly, a 0.5% solution of SBTI in 2.5 m*M* HCl was incubated at 90°C for 90 minutes for acid inactivation. A 0.25% solution of SBTI in 0.1 *N* NaOH was incubated for two hours at 36°C for alkali inactivation. Samples of inactivated inhibitor were dialyzed into FSW overnight at room temperature, and then diluted for experiments.

Determination of ID_{50} and P_{50}

To determine the inhibitor concentration which reduced enzyme activity to 50% of control levels (ID_{50}) or produced 50% polyspermy (P_{50}), regression curves were fit to the data points obtained for each of the inhibitors, and the 50% point calculated from the equation of line (Sokal and Rohlf, 1969). Coefficients of regression ranged from 0.93 to 0.99. Each experiment was repeated three times.

RESULTS

Consistent with previous observations (reviewed by Schuel, 1978, 1984), SBTI inhibited the purified egg protease (Fig. 1) and caused polyspermy (Fig. 2). In our hands the isolation procedure developed by Fodor *et al.* (1975) yielded a single serine

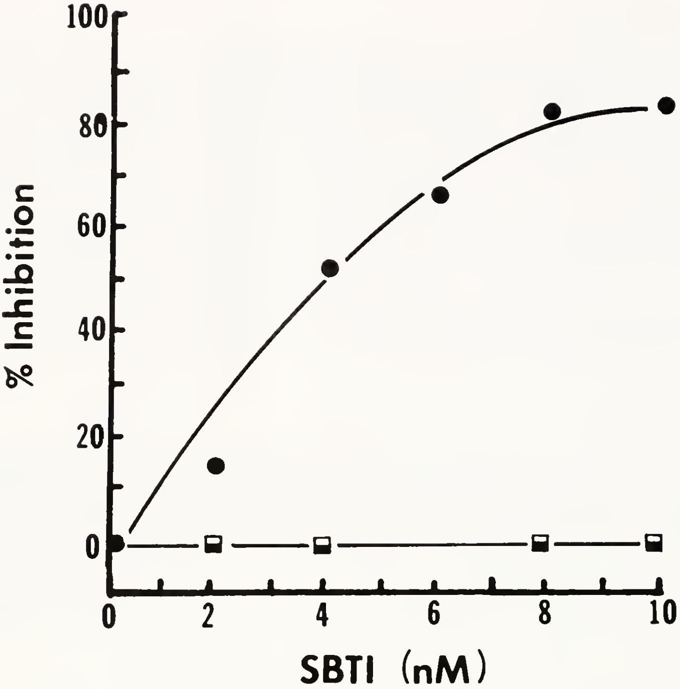

FIGURE 1. Effects of acid (☐) or alkali (■) pretreatment on the ability of native (●) SBTI to inhibit protease activity isolated from unfertilized *Strongylocentrotus* eggs as described under Methods.

protease with a specific activity against BAEE comparable to bovine pancreatic trypsin (Alliegro and Schuel, 1983a). Acid or alkali inactivation of SBTI according to Kunitz (1947) greatly reduced its inhibition of the egg protease (Fig. 1) *and* its capacity to promote polyspermy (Fig. 2). These treatments did not result in 100% inactivation of SBTI since some inhibition of the egg protease activity was observed when the concentration of SBTI in the enzyme assays was increased 100-fold (data not shown). This residual native SBTI could account for the slight incidence of polyspermy observed in experiments with inactivated SBTI (Fig. 2). These results clearly demonstrate that the induction of polyspermy by SBTI depends upon its inhibition of the egg protease.

We also examined the effects of other serine protease inhibitors on the purified egg protease and the induction of polyspermy (Table I). In addition to SBTI, ovomucoid, lima bean trypsin inhibitor, leupeptin, and antipain were found to be effective inhibitors of the egg protease. The relative potency of these inhibitors against the egg protease is similar to that found for trypsins and related serine proteases isolated from a wide variety of sources (reviewed by Kassel, 1970; Umezawa, 1976). All five inhibitors of the purified egg protease promoted polyspermy. Each of the inhibitors severely impaired elevation of the fertilization envelope, which exhibited a rosette appearance as originally described by Hagstrom (1956). The data also suggest a correlation between the *relative* effectiveness of these compounds as protease inhibitors and polyspermy promotors; the stronger inhibitors promoted polyspermy at lower concentrations. According to Spearman's rank correlation (Sokal and Rohlf, 1969),

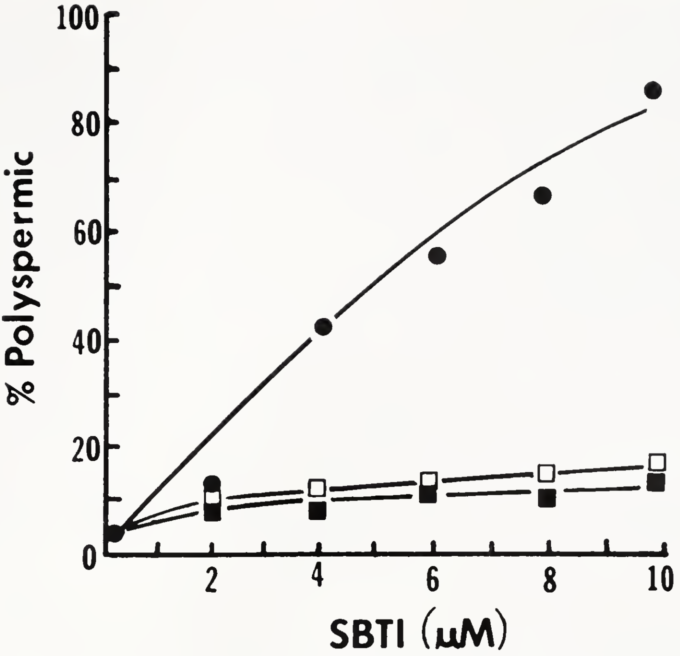

FIGURE 2. Effects of acid (□) or alkali (■) pretreatment on the ability of native (●) SBTI to cause polyspermy in *Arbacia* eggs as described under Methods.

$r_s = 0.90$. Thus, there is a statistical correlation between enzyme inhibition and polyspermy promotion by these serine protease inhibitors.

DISCUSSION

The question of specificity is a major concern in all studies involving the use of inhibitors. Past evidence for specificity in the induction of polyspermy by SBTI rested

TABLE I

Comparison of ID_{50} and P_{50} for inhibition of egg protease and promotion of polyspermy by serine protease inhibitors

Inhibitor	ID_{50} (*M*)	P_{50} (*M*)
Soybean trypsin inhibitor	6.01×10^{-9}	4.94×10^{-6}
Ovomucoid	1.06×10^{-6}	1.21×10^{-4}
Lima bean trypsin inhibitor	3.47×10^{-6}	1.42×10^{-4}
Leupeptin	4.22×10^{-6}	8.48×10^{-4}
Antipain	3.16×10^{-5}	3.69×10^{-4}

ID_{50} = the inhibitor concentration at which 50% of the enzyme activity purified from *S. purpuratus* eggs is abolished as described under Methods using BAEE as substrate.

P_{50} = the inhibitor concentration at which 50% polyspermy occurred in *A. punctulata* eggs, as described under Methods. The incidence of polyspermy for control eggs in these experiments was 3.3 + 0.58% (n = 15).

upon observations that it: inhibited the protease stored in cortical granules and secreted at fertilization; inhibited the serine protease purified from egg extracts or cortical granule exudates; and impaired fertilization envelope elevation. Furthermore, other serine protease inhibitors had similar effects (reviewed by: Runnstrom, 1966; Epel, 1978; Schuel, 1978, 1984). In fact, there appeared to be a relationship between their relative potencies as protease inhibitors and polyspermy promotors.

Nevertheless, Dunham *et al.* (1982) recently questioned the specificity of SBTI and other proteins as polyspermy promotors in sea urchins, and attributed their action to a non-specific protein effect. In our re-examination of this issue we found that acid or alkali inactivation of SBTI greatly diminishes its ability to inhibit the egg protease *and* to promote polyspermy. Thus, the protease inhibitory activity of SBTI is required for the protein to cause polyspermy. We have also confirmed previous observations that other serine protease inhibitors induce polyspermy in sea urchins (reviewed by Schuel, 1978, 1984). Furthermore, there is a correlation between their relative potencies as inhibitors of the egg protease and in their induction of polyspermy. Included in this group are antipain and leupeptin which are not proteins. Benzamidine and tosyl lysine chloromethyl ketone are other non-proteinaceous protease inhibitors known to induce polyspermy (Schuel *et al.,* 1973, 1976b). Based upon these results we conclude that the induction of polyspermy in sea urchins by protease inhibitors is due to a specific effect upon an egg protease.

Although the enzyme inhibition studies and polyspermy experiments were performed with different species, the correlation between the relative inhibitor potencies apparently holds true across species lines. These findings are consistent with previous observations using these inhibitors in *Arbacia, Strongylocentrotus,* and other species of sea urchins (Hagstrom, 1956; Vacquier *et al.,* 1972a, b; Schuel *et al.,* 1976a, b). This pattern emerges from the collective data in these earlier papers, but has not heretofore been presented in a single study performed under uniform conditions. That the correlation holds across species lines is not surprising, since a wide variety of trypsin-like proteases show a similar sensitivity profile with these inhibitors (Kassel, 1970; Umezawa, 1976). We did observe a difference in the absolute inhibitor concentrations required for the ID_{50} and P_{50} determinations. This is not surprising, considering the conditions of the two kinds of experiments. In the enzyme inhibition study, the inhibitor is competing with a synthetic substrate for a purified enzyme. In the biological experiment the inhibitor must compete effectively with natural substrates and under very different conditions than in the cuvette.

Dunham *et al.* (1982) reported that polyspermy can also be induced in sea urchins by catalase, superoxide dismutase, bovine serum albumin, and gelatin. These proteins are seemingly unrelated biochemically to SBTI or to each other. The authors hold this as evidence that the results obtained with these compounds are due to a non-specific protein effect. However, the chemical properties and existing evidence in support of specificity for each of these proteins must be carefully considered.

The function of catalase in aerobic systems is to remove and detoxify hydrogen peroxide (Chance, 1979). Sea urchin eggs produce H_2O_2 during fertilization (Foerder *et al.,* 1978; Boldt *et al.,* 1981). The fertilizing capacity of sea urchin sperm is significantly reduced by H_2O_2 (Evans, 1947; Boldt *et al.,* 1981; Coburn *et al.,* 1981). When catalase is added to fertilization cultures of *Arbacia,* the eggs become polyspermic (Coburn *et al.,* 1981; Dunham *et al.,* 1982). If the catalase is heat-denatured, the incidence of polyspermy is drastically reduced (Coburn *et al.,* 1981). Furthermore, catalase-induced polyspermy can be titrated away by the inclusion of 3-amino-1,2,4-triazole (Coburn *et al.,* 1981). Aminotriazole inhibits catalase by binding irreversibly to the complex formed between the enzyme and its substrate, H_2O_2 (Margoliash *et al.*, 1960; Chance *et al.*, 1979). These observations provide strong evidence that catalase induces polyspermy by the specific removal of H_2O_2.

The induction of polyspermy by superoxide dismutase reported by Dunham *et al.* (1982) is not an altogether unexpected finding in view of the facts that superoxide anion is produced along with H_2O_2 during the respiratory burst of somatic cells, that it is itself cytotoxic, and that it can react with H_2O_2 via the Haber-Weiss reaction to form other extremely cytotoxic oxygen radicals (Klebanoff, 1980). Since the addition of catalase and superoxide dismutase can prevent the production of toxic oxygen radicals by phagocytic cells (reviewed by Chance *et al.*, 1979; Klebanoff, 1980; Haliwell, 1982), it is possible that similar reactions may account for the promotion of polyspermy by these enzymes in sea urchins. If this were true, then the actions of these two proteins on sea urchin gametes would in fact be functionally interrelated.

Gelatin is a classic substrate used in the assay of proteases (Northrup and Hussey, 1923; Northrup, 1930). It was one of the substrates used in early studies of sea urchin egg proteases (Lundblad, 1954). *A priori*, one might expect that gelatin could cause polyspermy by acting as a competitive substrate for egg proteases. The low levels of polyspermy observed by Dunham *et al.* (1982) are consistent with this idea. Finally, bovine serum albumin did not cause polyspermy in our hands (Coburn *et al.*, 1981; Schuel and Schuel, 1981; Schuel, 1984). However, it is also a potential protease substrate, especially if denatured.

We believe that limited proteolysis, the respiratory burst, the possible production of arachidonic acid oxidation products, and other early responses in gamete interaction are intimately related events, as they are in somatic cells (Schuel, 1984). Under these circumstances, it is possible that the "multiple mechanisms" implicated in the polyspermy block are in fact related as steps in one or a few associated metabolic pathways. The variety of agents capable of promoting polyspermy may be acting at different points in a sequence of reactions, perhaps interrupting the generation of some particular product(s) critical to polyspermy prevention.

ACKNOWLEDGMENTS

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PURIFICATION AND CHARACTERIZATION OF MUSCLE PROTEINS FROM *APLYSIA CALIFORNICA*

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ABSTRACT

We report the purification and characterization of the major contractile proteins from body wall muscle of the marine mollusc *Aplysia californica,* and have found that they are quite similar to those of other molluscs. Myosin from this animal consists of heavy chains and at least two different classes of light chains; like the enzymatic activity of other molluscan myosins, its ATPase activity is regulated by Ca^{++}. Actin can be prepared from thin filaments by procedures that minimize denaturation of the protein. *Aplysia* tropomyosin consists of two subunits with apparent molecular weights of 33,000 and 37,000. Paramyosin migrates as a polypeptide of molecular weight 100,000. A description of the procedures for purifying these proteins from *Aplysia* may be useful to neurobiologists who are now using this animal extensively.

INTRODUCTION

Invertebrate muscle proteins have been intensively studied because of the insight they provide into the mechanisms that regulate muscle contraction. Scallop muscle is a particularly suitable source of material because it is available in large amounts as a homogeneous tissue (Szent-Györgyi *et al.,* 1973). Studies of molluscan muscle show that contraction is triggered by binding of Ca^{++} to myosin in thick filaments, rather than to troponin in thin filaments as in vertebrate muscle (Lehman and Szent-Györgyi, 1975). These studies have also demonstrated that a distinctive component of invertebrate muscle is paramyosin, a protein whose function is not yet clear, but which is thought to serve a structural role in thick filaments (Szent-Györgyi *et al.,* 1971).

During the past decade, contractile proteins have also received considerable attention from cell biologists interested in the mechanism of motility in non-muscle cells (see, for example, Taylor and Condeelis, 1979; Bray and Gilbert, 1981; Brinkley, 1982). Our interest concerns the possible participation of actin and myosin in fast axoplasmic transport, a form of intracellular motility in which synaptic vesicles and other membranous organelles are moved along the axon of a nerve cell to its terminals (Schwartz, 1979; Grafstein and Forman, 1980). Our experimental strategy is to microinject anti-actin antibodies and other binding proteins into giant neurons of the marine mollusc, *Aplysia* (Goldberg *et al.,* 1980; Goldberg, 1982). To obtain sufficient quantities of the contractile proteins to raise antibodies, we isolated actin, myosin, tropomyosin, and paramyosin from *Aplysia* muscle. Here we report the purification

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Abbreviations: HMM = heavy meromyosin; LMM = light meromyosin; SDS = sodium dodecyl sulfate.

and characterization of these proteins. Biochemical studies of *Aplysia* actin are of special interest because the antibody raised against the purified protein has unusual specificity: it cross-reacts only with cytoplasmic actin but not with sarcomeric actin from vertebrates (Lubit and Schwartz, 1980; Hall *et al.,* 1981; Lubit and Schwartz, 1983).

MATERIALS AND METHODS

Muscle from body wall, buccal mass, and heart was obtained from *Aplysia californica* purchased from Pacific Bio-Marine, Venice, California, or raised at the *Aplysia* Mariculture Facility at the Marine Biological Laboratory, Woods Hole, Massachusetts. Animals were extended with their ventral surface up, pinned rostrally and caudally, and opened with an incision along the entire length of the foot. The viscera were removed, and the flaps of body wall stretched and pinned laterally until taut. The buccal mass was removed, cleaned of the buccal ganglion and attached nerves, and the red buccal muscle dissected from the radula. The heart was freed from the pericardium. Muscle from the body wall was most efficiently obtained by separating it as a single strip from the skin using a sharp scalpel. Dissection began laterally and proceeded toward the midline. As the muscle was freed from the skin it contracted into a thick band on each side of the animal. These bands were cut at their ends and peeled away from the carcass.

Muscle tissue was rinsed briefly at 0°C in 40 m*M* NaCl, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 5 m*M* sodium phosphate (pH 7.0), and was either used immediately or stored at −20°C in 50% glycerol. Glycerinated muscle was stable for six months, after which significant proteolysis of contractile proteins (especially myosin) occurred, and recovery of purified proteins was low.

Myosin and meromyosins

Myosin was prepared by ammonium sulfate fractionation of actomyosin as described by Chantler and Szent-Györgyi (1978), and was purified further by gel filtration on a column (1.5 × 85 cm) of Sepharose 4B-CL (Pharmacia, Piscataway, New Jersey) in 0.6 *M* NaCl, 0.2 m*M* dithiothreitol, 1 m*M* sodium pyrophosphate, and 5 m*M* sodium phosphate (pH 7.0). It was stored either at 4°C as a suspension in 65% ammonium sulfate, or at −20°C in 50% glycerol. Myosin from rabbit skeletal muscle was prepared by the procedure of Mommaerts and Parrish (1951).

To prepare heavy and light meromyosins, myosin from body wall muscle (2–25 mg/ml) was digested with 0.2% by weight of trypsin (Type DCC, Miles Laboratories, Elkhart, Indiana) at 25°C in 0.5 *M* KCl and 30 m*M* Tris-HCl (pH 8.2) (Balint *et al.,* 1975); 1 m*M* $MgCl_2$ was also present (Craig *et al.,* 1980). Digestion was terminated by the addition of soybean trypsin inhibitor (Type I-S, Sigma Chemical Co., St Louis, Missouri) equal to twice the weight of trypsin. The reaction mixture was dialyzed against 20 m*M* NaCl, 1 mm $MgCl_2$, 0.1 m*M* EGTA, 0.1 m*M* dithiothreitol, 5 m*M* sodium phosphate (pH 7.0), and was centrifuged at 30,000 × *g* for 30 min to remove the precipitate of light meromyosin (LMM) and undigested myosin. The supernatant contained heavy meromyosin (HMM). The precipitate was washed several times with 5 m*M* sodium phosphate (pH 6.5), and LMM purified from it by ethanol fractionation (Lowey *et al.,* 1969).

Thin filaments and actin

Thin filaments were prepared by extracting myofibrils at pH 6 (Szent-Györgyi *et al.,* 1971). Actin was purified from thin filaments by two methods: (1) application of filaments to a column (2.5 × 30 cm) of DEAE-cellulose, followed by elution with a KCl gradient using the conditions of Gordon *et al.* (1976); actin-containing fractions were combined, and the protein polymerized and sedimented as described (Gordon *et al.*, 1976); (2) extraction of an acetone powder (Szent-Györgyi, 1951) of thin filaments with 0.5 m*M* 2-mercaptoethanol (pH 6–6.5) for 30 min at 25°C; after addition of 10 m*M* $MgCl_2$, the extract was kept at 25°C for another 1.5 h, and F-actin was collected by centrifugation at 100,000 × *g* for 3 h.

Actins from rabbit skeletal muscle and chicken gizzard were prepared by the method of Spudich and Watt (1971).

To examine thin filaments by electron microscopy, 0.1 mg/ml samples were applied to Formvar-carbon-coated grids, negatively stained with 1% uranyl acetate, and viewed in a Philips 301 electron microscope.

Tropomyosin and paramyosin

Tropomyosin was obtained during chromatography of thin filaments on DEAE cellulose (see above, method 1). Fractions containing tropomyosin were made 10 m*M* in $MgCl_2$ and centrifuged at 100,000 × *g* for 5 h to remove actin. Tropomyosin was also extracted along with actin from the acetone powder of thin filaments (see above, method 2). The supernatant remaining after sedimentation of F-actin was enriched in tropomyosin.

Paramyosin was isolated by the procedure of Szent-Györgyi *et al.* (1971), and purified further by addition of ice-cold acetone and extraction of the dried acetone powder at 25°C with 0.1 *M* NaCl and 5 m*M* sodium phosphate (pH 7.4). The extract was again precipitated with acetone and extracted once more with the same buffer.

Immunological procedures

To raise antibodies against intact myosin (heavy and light chains), the purified protein from body wall muscle was electrophoresed on 5% polyacrylamide gels in the absence of sodium dodecyl sulfate (SDS). The gels were stained with Coomassie Blue, and the region of the gel containing myosin was cut out, lyophilized, and powdered. The powder was suspended in 0.85% NaCl using a Teflon-glass homogenizer and was then emulsified with one-half volume of Freund's complete adjuvant (Gibco, Grand Island, New York). New Zealand white rabbits were injected intramuscularly in their hind legs with emulsion containing 20–25 μg of myosin once per week for four weeks, and were boosted monthly thereafter with the same amount of immunogen. Rabbits were bled weekly beginning one week after the initial series of injections. Immunoglobulin was prepared from antisera by fractionation with ammonium sulfate at 50% saturation.

To raise antibodies against heavy and light chains separately, the purified protein was electrophoresed in polyacrylamide gels containing SDS, and the regions of the stained gels containing the subunits of myosin were excised and prepared as above. Light chains of both high and low molecular weight were used together for immunization.

Double immunodiffusion on Ouchterlony plates was carried out using glass microscope slides covered with 1% agarose (type I, Sigma) in 20 m*M* sodium pyrophosphate (pH 8.6). Plates were kept in a moist chamber at 25°C for 1–3 days, and were then washed, dried, and stained with Coomassie Blue.

Analytical procedures

Electrophoresis on polyacrylamide gels was carried out with SDS by the method of Laemmli (1970) or of Weber and Osborne (1969). Gradient slab gels of 4–30%

polyacrylamide were prepared using the gel and electrode buffers of Weber and Osborne (1969).

ATPase was assayed at 25°C by measuring the amount of P_i released using the method of Taussky and Schorr (1953). In some experiments, ATP hydrolysis was calculated from the rate of proton release using a pH-stat with an automatic titrator (Radiometer, Copenhagen).

Protein was assayed colorimetrically either by the method of Bradford (1976) or of Lowry *et al.* (1951), using bovine gamma globulin as standard.

RESULTS

Myosin

Myosin from body wall muscle of *Aplysia* was prepared using Chantler and Szent-Györgyi's (1978) procedure for isolating other invertebrate myosins (Fig. 1). Extraction

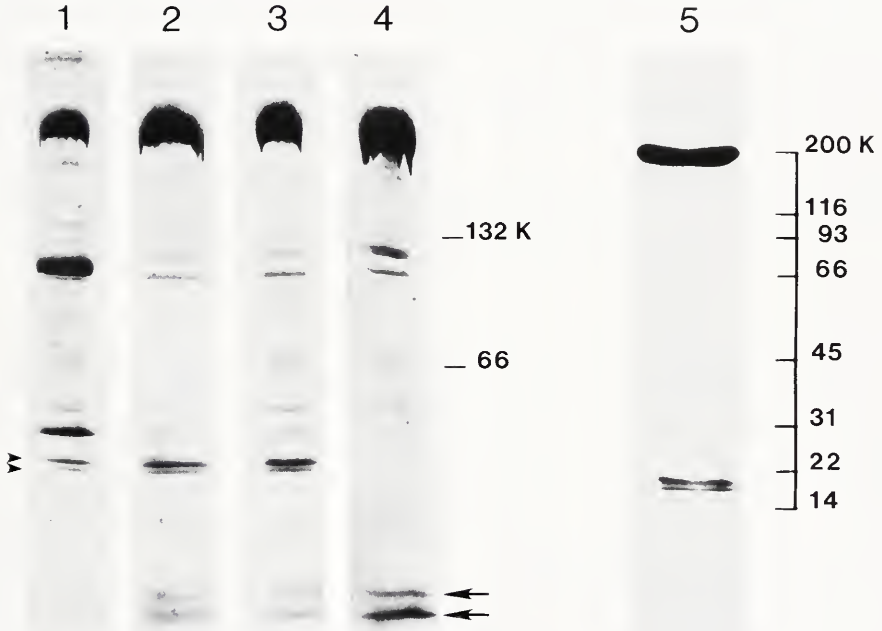

FIGURE 1. Analysis of proteins obtained during the purification of myosin from *Aplysia* body wall muscle. Samples were analyzed by electrophoresis in SDS on gradient slab gels of 4–30% polyacrylamide (lanes 1–4) or on 9% polyacrylamide slab gels (lane 5). The molecular weight markers for lanes 1–4 are monomeric (66,000) and dimeric (132,000) bovine serum albumin; the markers for lane 5 are rabbit myosin (200,000), beta-galactosidase (116,000), phosphorylase b (93,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (22,000), and lysozyme (14,000).

Lane 1: Actomyosin obtained by extraction of myofibrils with 0.6 *M* NaCl and 5 m*M* ATP (10 μg protein). The arrowheads indicate the positions of the two subunits of tropomyosin. Lane 2: Supernatant obtained after fractionation of actomyosin with ammonium sulfate at 40% saturation (20 μg). Lane 3: Myosin precipitated by further fractionation with ammonium sulfate to 65% saturation (10 μg). Lane 4: Myosin after precipitation at low ionic-strength and washing (22 μg). The arrows indicate the positions of the two classes of myosin light chains. Lane 5: Peak fractions after gel filtration of myosin on Sepharose 4B-CL (20 μg). Two bands are visible in the region between the 22,000 and 14,000 molecular weight markers, but the lower band is an artifact of electrophoresis and was present even in gel lanes that did not contain protein.

of myofibrils with 0.6 *M* NaCl and 5 m*M* ATP at pH 7 solubilizes myosin, paramyosin, actin, and tropomyosin (lane 1). Addition of ammonium sulfate to 40% saturation removes most of the paramyosin and actin (lane 2). Further addition of ammonium sulfate to 65% saturation precipitates myosin, which can be solubilized again (lane 3) and then freed of tropomyosin by precipitation at low ionic strength and washing. The final preparation (lane 4) consists principally of myosin, but still contains small amounts of other proteins. These contaminants could be almost completely removed by gel filtration on a column of Sepharose 4B-CL (Fig. 1, lane 5). In six independent preparations, recovery of gel-filtered myosin ranged from 2.4–3.4 mg per gram wet-weight of muscle.

Aplysia myosin, like other vertebrate and invertebrate myosins, consists of heavy and light chains. On SDS-polyacrylamide gels (Laemmli, 1970), the heavy chain migrates with an apparent molecular weight of 200,000 and the light chains appear as a single, diffuse band with an apparent molecular weight of 19,000 (Fig. 1, lane 5). In gradient gels, the light chains can be resolved into two components with molecular weights of 18,500 and 19,000 (Fig. 1, lane 4). The 18,500 molecular weight component stains more darkly with Coomassie Blue, and presumably is present in greater molar amounts.

To investigate whether *Aplysia* myosin is immunologically related to vertebrate myosins, we raised antisera in rabbits against the intact *Aplysia* protein, as well as against its heavy and light chains separately. Although they all gave a single, sharp precipitin line with both purified *Aplysia* myosin and a crude extract of body wall muscle, none of these antisera were found to cross-react during double-immunodiffusion with myosin from rabbit skeletal muscle (Fig. 2). The antisera also did not cross-react with actomyosin from chicken gizzard, or with actin from body wall muscle of *Aplysia*, rabbit skeletal muscle, or chicken gizzard.

ATPase activity of myosin and meromyosins

The ATPase activity of myosin purified from *Aplysia* body wall muscle is inhibited by Mg^{++} and activated by Ca^{++} and K^+/EDTA (Table I), features that are typical of myosins from both vertebrate and invertebrate sources. In addition, the ATPase activity of *Aplysia* myosin is stimulated ten-fold by purified actin, but only in the presence of Ca^{++} (Table I). This dependence on Ca^{++} is characteristic of myosins from molluscan species that display thick filament-linked regulation of contraction (Lehman and Szent-Györgyi, 1975).

Heavy and light meromyosins can be prepared from *Aplysia* myosin by a three-minute digestion with trypsin. As expected from studies with meromyosins from rabbit skeletal muscle, the ATPase activity of *Aplysia* myosin resides entirely in the HMM portion of the molecule: in the presence of Ca^{++}, HMM had an activity of 0.49 μmoles P_i/min/mg, compared to LMM with an activity of 0.01 (for assay conditions, see legend to Table I).

Thin filaments and actin

Thin filaments can be prepared from body wall muscle of *Aplysia* using the method of Szent-Györgyi *et al.* (1971), in which myofibrils are extracted at pH 6 with ATP and EDTA. Electron microscopic examination reveals filaments with a diameter of 6 nm and a range of different lengths (Fig. 3). We did not observe any dense bodies attached to the ends of filaments (Szent-Györgyi *et al.*, (1971). Actin, with an apparent molecular weight of 43,000, is the major protein in this preparation, although other proteins are also present (Fig. 4, lane 1). Some of these proteins,

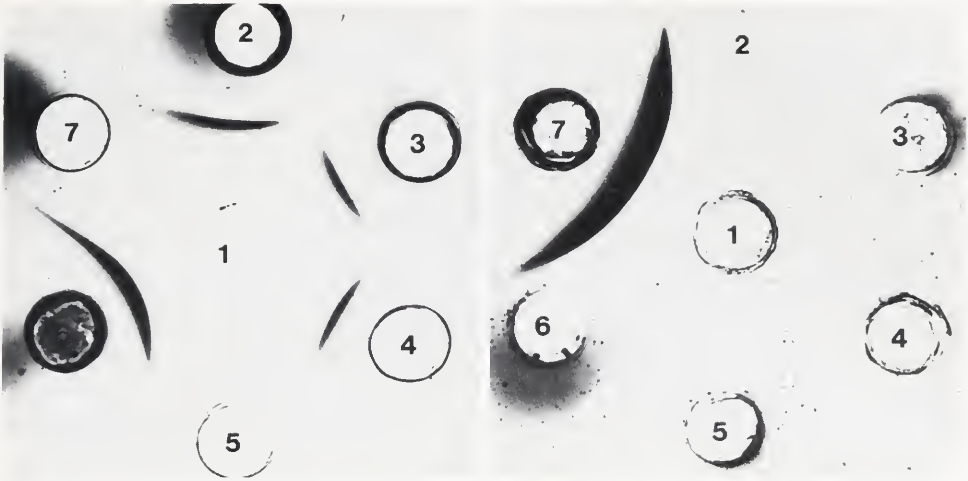

FIGURE 2. Double immunodiffusion using antiserum raised against intact myosin from *Aplysia* body wall muscle. Ouchterlony plates were washed, dried, and stained with Coomassie Blue. The wells contained:

Left: (1) immunoglobulin fraction of antiserum (2 mg/ml), (2–5) myosin purified from body wall muscle (0.2–1.4 mg/ml), (6) crude extract of body wall muscle in 0.6 *M* NaCl (8 mg/ml), and (7) rabbit skeletal myosin (1.4 mg/ml).

Right: (1) immunoglobulin fraction of antiserum (2 mg/ml), (2) rabbit skeletal actin (3.8 mg/ml), (3) actin from body wall muscle (1.5 mg/ml), (4) actin from chicken gizzard smooth muscle (1.5 mg/ml), (5) actomyosin extracted from chicken gizzard myofibrils in 0.6 *M* NaCl (8 mg/ml), (6) rabbit skeletal myosin (1.4 mg/ml), and (7) extract of body wall muscle in 0.6 *M* NaCl (8 mg/ml).

including tropomyosin, sediment with actin after centrifugation of filaments at 100,000 × *g* for 3 h (not shown). Typically, the overall yield of protein in thin filament preparations was 1 mg per gram wet-weight of muscle. Thin filaments could also be prepared from buccal and heart muscle (not shown).

TABLE I

ATPase Activity of Aplysia *myosin*

Myosin alone[1]	Activity[2]
Ca^{++}	0.35
Mg^{++}	0.02
K^{+}/EDTA	0.37
Effect of actin[3]	
Actin, Ca^{++}	0.22
Actin, EGTA	0.06
No actin, Ca^{++}	0.02

[1] The assay mixtures contained 5 m*M* $CaCl_2$/0.6 *M* NaCl; 5 m*M* $MgCl_2$/0.1 m*M* EGTA/0.6 *M* NaCl; or 2 m*M* EDTA/0.6 *M* KCl.

[2] Assays were conducted at pH 7.5 in 10 m*M* imidazole-HCl buffer containing 1 m*M* ATP. ATPase activity is expressed as μmoles P_i/min/mg protein and was determined after a 10 min incubation at 25°C; the rate of P_i release during this time was found to be constant. Values are typical, and represent the average of duplicate determinations. Similar values were obtained with at least two and as many as four independent preparations of *Aplysia* myosin. Assays were carried out over a period of six months with little variation.

[3] The assay mixture contained 0.35 m*M* $CaCl_2$/0.35 m*M* $MgCl_2$/30 m*M* NaCl; or 0.1 m*M* EGTA/0.35 m*M* $MgCl_2$/30 m*M* NaCl. Rabbit F-actin was added at a ratio of 0.5–0.7 mg/mg of myosin.

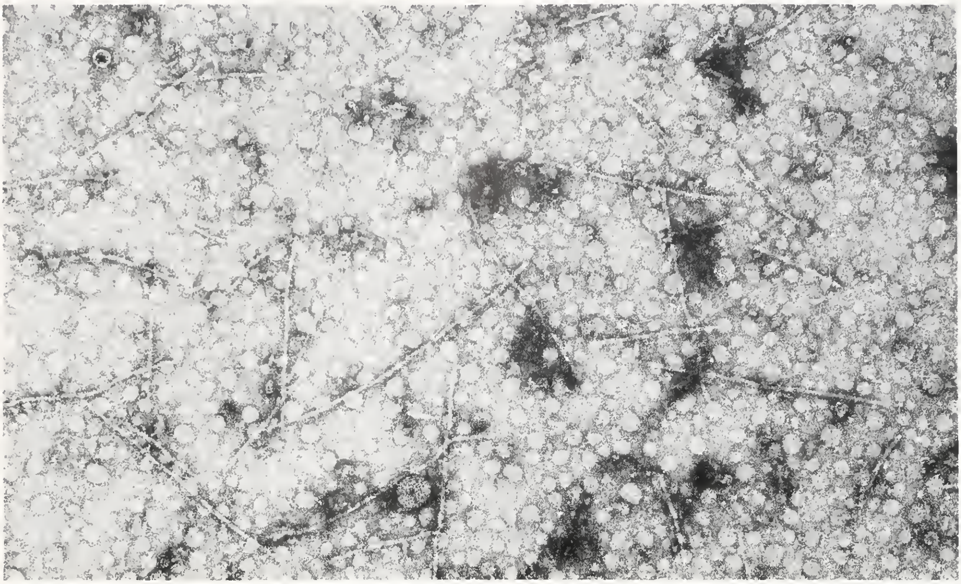

FIGURE 3. Electron micrograph of thin filaments from *Aplysia* body wall muscle. Magnification 77,000×. The filaments, negatively stained with 1% uranyl acetate, are seen against a background of droplets of stain.

We purified actin from *Aplysia* thin filaments by two methods. The first, chromatography on DEAE-cellulose (Gordon *et al.*, 1976), seemed advantageous because we found that *Aplysia* actin is denatured during the successive cycles of polymerization and depolymerization that are required in other purification procedures (Spudich and Watt, 1971). When a gradient of KCl was applied to the ion-exchange resin, protein was eluted in several peaks (Fig. 5). Electrophoretic analysis of these fractions showed that actin was eluted between 0.22 and 0.26 M KCl (Fig. 4, lanes 2–4). After the actin-containing fractions were combined and the protein polymerized by addition of 2 mM $MgCl_2$, essentially pure F-actin free of tropomyosin could be collected by centrifugation at 100,000 × g for 5 h (not shown). Typically, 0.5 mg of actin could be obtained per mg of thin filaments. Tropomyosin was eluted at higher salt concentrations (see below).

In the second method, actin was extracted from an acetone powder of thin filaments and then polymerized by the addition of $MgCl_2$. The F-actin collected by centrifugation was still contaminated with a small amount of tropomyosin (Fig. 4, lane 7).

Tropomyosin

Fractions eluting between 0.28 and 0.30 M KCl during chromatography of thin filaments on DEAE-cellulose contained tropomyosin contaminated by only traces of actin (Fig. 4, lanes 5, 6). Addition of $MgCl_2$ and centrifugation at high speed removed most of the actin (not shown). 0.2 mg of purified tropomyosin was obtained per mg of thin filaments. Relatively pure tropomyosin also could be obtained in the supernatant that remained after collection of F-actin from the extract of an acetone powder of thin filaments (Fig. 6, lane 1). On 5% SDS-polyacrylamide gels, tropomyosin appeared as a single, broad band with a molecular weight of 35,000 (Fig. 6). On 4–30% gradient gels, it could be resolved into two subunits with apparent molecular weights of 33,000 and 37,000 (Fig. 1, lane 1).

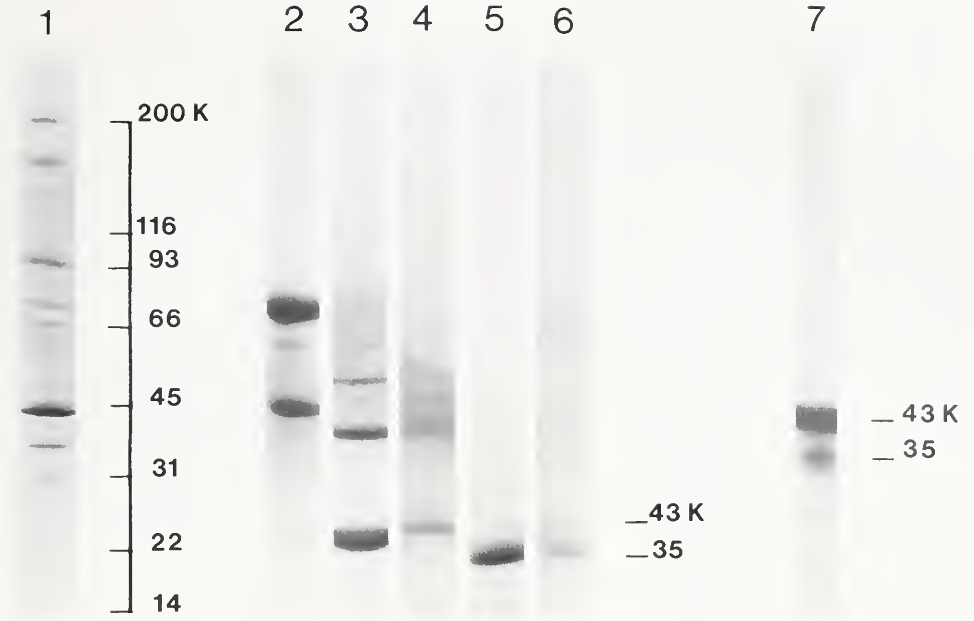

FIGURE 4. Analysis of thin filaments and actin from *Aplysia* body wall muscle. Samples were analyzed by electrophoresis in SDS on gradient slab gels of 4–30% polyacrylamide (lanes 1–6) or on a 5% polyacrylamide tube gel (lane 7). The molecular weight markers for lane 1 are the same as for lane 5 of Figure 1; for lanes 2–7 the markers are actin (43,000) and tropomyosin (35,000) from rabbit skeletal muscle.

Lane 1: thin filaments (15 μg protein), lanes 2–6: fifty μl samples of the fractions eluted during DEAE-cellulose chromatography of thin filaments described in the legend to Figure 5; lane 2, fraction no. 39; lane 3, fraction no. 42; lane 4, fraction no. 46; lane 5, fraction no. 49; lane 6, fraction no. 53; lane 7: actin purified from an acetone powder of thin filaments (20 μg).

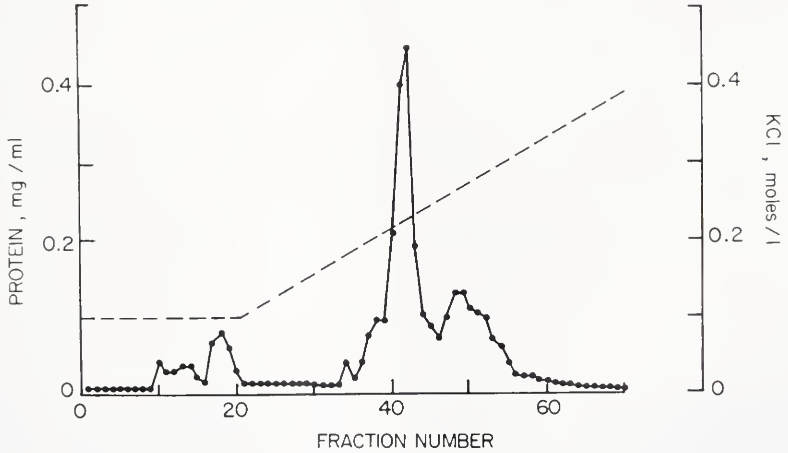

FIGURE 5. Chromatography of thin filaments from *Aplysia* body wall muscle on DEAE-cellulose. The column (2.5 × 30 cm) was equilibrated with 0.1 *M* KCl in Buffer D of Gordon *et al.*, 1976 (10 m*M* imidazole-Cl (pH 7.5), 0.1 m*M* $CaCl_2$, 0.5 m*M* ATP, 0.75 m*M* beta-mercaptoethanol). 25 ml of Buffer G (same as Buffer D, but with 3 m*M* imidazole-Cl) was then applied, followed by 50 mg of thin filaments (0.3 mg/ml), and then 25 ml more of Buffer G. The column was then eluted with 125 ml of 0.1 *M* KCl in Buffer D and then a linear gradient (1 liter) of 0.1 *M* to 0.5 *M* KCl in Buffer D. Fractions of 17 ml were collected; ——, protein, – – –, concentration of KCl.

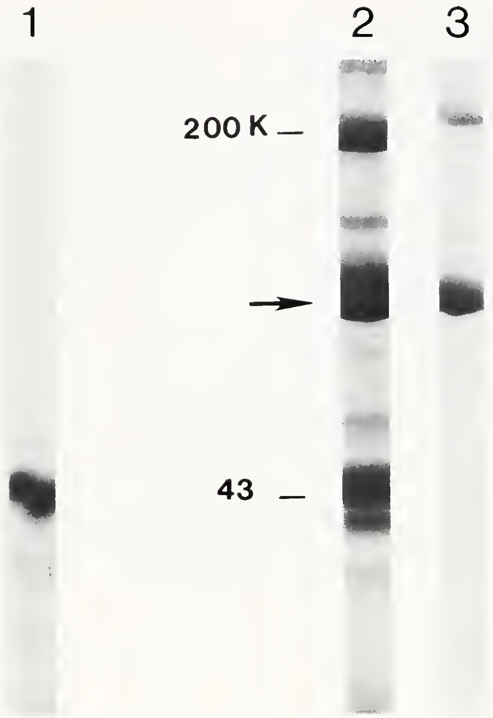

FIGURE 6. Analysis of tropomyosin and paramyosin from *Aplysia* body wall muscle. Samples were analyzed by electrophoresis in SDS on 5% polyacrylamide tube gels. Molecular weight markers for lanes 2 and 3 are myosin heavy chain (M_r 200,000) and actin (M_r 43,000), both from rabbit skeletal muscle.

Lane 1: Tropomyosin purified from an acetone powder of thin filaments [15 μg; the protein comigrated with tropomyosin from rabbit skeletal muscle (M_r 35,000)]; lane 2: paramyosin (arrow) prepared by differential extraction of thick filaments (25 μg); and lane 3: paramyosin purified further by acetone precipitation (15 μg). A small amount of myosin heavy chain remains.

Paramyosin

Paramyosin prepared from body wall muscle by differential extraction of thick filaments (Szent-Györgyi *et al.*, 1971) was contaminated with myosin and actin (Fig. 6, lane 2). After addition of acetone and extraction at pH 7.4 two times, the preparation was highly enriched in paramyosin (Fig. 6, lane 3), which had a molecular weight of 100,000.

DISCUSSION

Myosin

Aplysia myosin, purified from body wall muscle by ammonium sulfate fractionation (Chantler and Szent-Györgyi, 1978) followed by gel filtration, is similar to myosins from other molluscan species. The purified protein consists of heavy chains of molecular weight 200,000 and two classes of light chains with molecular weights of 18,500 and 19,000. Szent-Györgyi *et al.* (1973) have found that scallop myosin also contains heavy chains of molecular weight 200,000 and light chains of molecular weight 18,000. Scallop light chains can be resolved into two components by electrophoresis on urea-polyacrylamide gels. One type of light chain can be selectively removed from myosin by EDTA and is responsible for conferring Ca^{++}-sensitivity on the actin-activated ATPase activity of the molecule. The other light chain contains cysteine residues and its function is unknown. There are two light chains of each type in a molecule of scallop myosin (Kendrick-Jones *et al.,* 1976).

Although vertebrate skeletal myosin also contains both heavy and light chains, these subunits appear to be functionally and structurally distinct from their molluscan counterparts (Kendrick-Jones *et al.,* 1976). Consistent with these differences, we have found that antibodies raised against *Aplysia* myosin, as well as against its separated heavy and light chains, do not cross-react with the myosins from chicken gizzard or rabbit skeletal muscle.

Contraction of vertebrate skeletal muscle is triggered by binding of Ca^{++} to troponin-containing thin filaments. In contrast, Lehman and Szent-Györgyi (1975) have shown that binding of Ca^{++} to myosin in thick filaments initiates contraction in molluscan muscle. One biochemical correlate of myosin-linked regulation is the ability of rabbit actin to stimulate the ATPase activity of molluscan myosin in the presence, but not in the absence, of Ca^{++}. By this criterion, *Aplysia* muscle, like that of other molluscs, displays myosin-linked regulation; when Ca^{++} is omitted, the actin-stimulated ATPase activity of *Aplysia* myosin is reduced by 74% (Table I). In the same assay, the ATPase activity of rabbit myosin is slightly greater when Ca^{++} is absent (data not shown).

Thin filaments and actin

Thin filaments can be prepared from body wall muscle of *Aplysia* by extraction of myofibrils with ATP and EDTA. We chose to purify actin from thin filaments, rather than from an acetone powder of myosin-depleted whole muscle (Spudich and Watt, 1971), because we found that the yield of actin using the latter procedure was poor. One reason for the low yield may be that *Aplysia* actin was easily denatured during the polymerization at high ionic strength that is used in the procedure of Spudich and Watt. Purified *Aplysia* actin was prepared by chromatography of thin filaments on DEAE-cellulose (Gordon *et al.,* 1976), or by extraction of an acetone powder of thin filaments. We also have purified denatured *Aplysia* actin by preparative SDS gel electrophoresis (Sherbany and Schwartz, unpub.), and have used it as an immunogen to raise an antiserum that cross-reacts specifically with vertebrate cytoplasmic actin (Lubit and Schwartz, 1980, 1983).

Tropomyosin and paramyosin

Tropomyosin from body wall muscle of *Aplysia,* like vertebrate skeletal tropomyosin, consists of two subunits that migrate on polyacrylamide gels with apparent molecular weights of 33,000 and 37,000. *Aplysia* paramyosin has a molecular weight of about 100,000, and is of considerable interest because it is the major phosphoprotein in muscle. Stimulation of the identified serotonergic giant cerebral neuron or application of serotonin causes an elevation in the synthesis of cAMP within buccal muscle (Weiss *et al.,* 1979), and this, in turn, leads to the increased phosphorylation of paramyosin (Weiss *et al.,* 1981). Application of serotonin also promotes the phosphorylation of paramyosin in other *Aplysia* muscles as well as in nervous tissue (Katz *et al.,* 1983). Work is now in progress to determine whether the paramyosin found in extracts of nervous tissue is a contaminant from the muscular connective tissue sheath of the ganglia or a native constituent of nerve cells.

ACKNOWLEDGMENTS

We wish to thank Dr. Andrew G. Szent-Györgyi for his help in performing the first ATPase determinations with *Aplysia* muscle in his laboratory at the MBL and for helpful discussions of the biochemistry of molluscan muscle. We also thank Alice Elste for assistance in preparing the electron micrograph. This research was supported by research grants NS 19332 (from the N.I.H.) and BNS 8215311 (from the N.S.F.) to J.H.S. The other two authors were U. S. Public Health Service Medical Scientist Trainees (Grant GM-07367 to D.A.H., and GMO-1668 and GMO-7308 to A.A.S.).

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MULTIPLE PATTERNS OF DEVELOPMENT IN *STREBLOSPIO BENEDICTI* WEBSTER (SPIONIDAE) FROM THREE COASTS OF NORTH AMERICA

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ABSTRACT

Streblospio benedicti Webster, a small tube-dwelling polychaete common in Pacific, Gulf of Mexico, and Atlantic estuaries of North America, exhibits both lecithotrophic and planktotrophic modes of larval development. In lecithotrophic forms females produce few (9–50) large ova (100–200 μm diam.). These develop in dorsal pouches into 9–12 setiger larvae, competent to settle at release. Females of planktotrophic forms produce large broods (100–548) of small ova (70–90 μm), brood larvae in dorsal pouches or beneath dorsal branchiae, and release 3–7 setiger larvae which bear long swimming setae and feed in the plankton for 1–5 weeks before settling. Lecithotrophy is reported for *S. benedicti* populations on all three coasts of N. America, planktotrophy from the Atlantic and Gulf coasts only. Reproductive differences observed in the field are maintained by laboratory cultures reared under constant (20°C) conditions, though individuals from planktotrophic and lecithotrophic populations are interfertile. Developmental variations observed in the field are believed to generate different patterns of dispersal, recruitment, population growth (r), and mortality. Poicilogony, the occurrence of multiple development modes, may account for the considerable success of *S. benedicti* in N. America.

INTRODUCTION

This paper describes the occurrence of both planktotrophic (feeding) and lecithotrophic (non-feeding) modes of development in the estuarine polychaete *Streblospio benedicti*. The dichotomy between these development modes in marine invertebrates, and between associated variations in egg size, fecundity, and length of planktonic larval life, have generated interest among developmental biologists, zoologists, and ecologists for many years (Thorson, 1946, 1950). These traits and their ecological implications are examined here for a single species.

The adaptive nature of marine invertebrate trophic modes remains a mystery despite theoretical efforts to model the coexistence of reproductive strategies (Vance, 1973a, b; Christiansen and Fenchel, 1979; Pechenik, 1979; Caswell, 1981) and an empirical search for energetic correlates of alternate development patterns (Hines, 1979; Spight, 1979; Todd, 1979; Hughes and Roberts, 1980; Grahame, 1982; Defreese and Clark, 1983). Clues to the adaptive differences between planktotrophy and lecithotrophy may be found in the study of poicilogonous species, those species which exhibit multiple reproductive modes. Such variability is especially common among the Spionidae (Polychaeta), in which brood protection and nurse egg feeding (adelphophagy) allow considerable flexibility in the duration of the planktonic larval phase and in the mode of nutrition. Multiple patterns of larval development have been

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described in *Pygospio elegens* (Rasmussen, 1973), *Spio setosa* (Simon, 1968), *Polydora quadrilobata* (Blake, 1969), and *Boccardia proboscidea* (Blake and Kudenov, 1981). However, none of these investigations has shown a single female to produce both planktotrophic and lecithotrophic (non adelphophagic) larvae, and crossing experiments have not been conducted to eliminate the possibility of speciation. In several instances close examination of apparently poicilogonous polychaetes revealed the existence of multiple sibling species (Grassle and Grassle, 1976; Christie, 1982).

The study organism

Streblospio benedicti (Spionidae) is a small (≤20 mm) infaunal polychaete that lives in the top few cm of muddy sediments and is a deposit and suspension feeder at the sediment-water interface. It is common in wetlands and estuaries throughout the Pacific, Gulf, and Atlantic coasts of North America and has also been reported from northern Europe, the Mediterranean Sea, the Black Sea, and Venezuela (Carlton, 1979).

Streblospio benedicti is best known as an opportunist which colonizes stressed or organically enriched sediments (Grassle and Grassle, 1974; Pearson and Rosenberg, 1978). *Streblospio* responds positively to structures on the mud surface such as artificial tubes (Dauer *et al.,* 1982), cages (Virnstein, 1977, 1978), settling containers (McCall, 1977; Levin, in press), or pits (Levin, in press) and has played a key role in studies of: meiofaunal-macrofaunal interactions in marshes (Bell and Coull, 1980; Watzin, 1983; and pers. comm.); predation in shallow infaunal communities (Quammen, 1981; Virnstein, 1977); competition among infauna (Levin, 1981, 1982a; R. Whitlatch, pers. comm.); and mesocosm dynamics (J. F. and J. P. Grassle, pers. comm., L. Watling, pers. comm.).

Though the reproductive characteristics of *S. benedicti* are important to the interpretations of all of these investigations, existing descriptions of development are spotty and conflicting. Larval development of *S. benedicti* has been described briefly by Campbell (1957) for Cape Cod (Massachusetts) populations and by Dean (1965) for Mystic River Estuary (Connecticut) populations; in both cases development was planktotrophic. Lecithotrophic development in west coast populations was discussed by Blake (1965) for Morro Bay, California and Levin (1982b) for Mission Bay, California. No previous descriptions of development are known for populations in the Gulf of Mexico.

MATERIALS AND METHODS

Sediment samples were collected by hand from 3 intertidal mudflats in California (Tijuana Slough, Dec. '82; Mission Bay, Nov. '82–April '83; and Elkhorn Slough, March '83), from tidal *Spartina* marshes in Texas (Big Slough, June and Oct. '83), Georgia (Sapelo Island, Nov. '83), and North Carolina (Tar Landing, Feb. and Oct. '83), and from shallow brackish water bays in Texas (Copano Bay, Oct. '83) and Florida (Sebastian River, March '83) (Fig. 1). Subtidal collections were also made in New Bedford Harbor, Massachusetts with a Van Veen grab (Oct.–Dec. '82) and from 6 m high cylindrical tanks at the Marine Experimental Research Laboratory (MERL) in Narragansett Bay, Rhode Island (Nov. '82–Aug. '83) with a pole-mounted core ($5 \text{ cm}^2 \times 4$ cm depth). Salinities at all sites ranged from 28–34‰ except at Copano Bay (2‰) and Sabastian River (≈5‰).

Sediment samples were sieved through 300 or 500 μm screens and specimens of *Streblospio* were isolated for observation and initiation of laboratory cultures. Polychaetes were maintained at room temperature (20–23°C) in glass crystalizing dishes

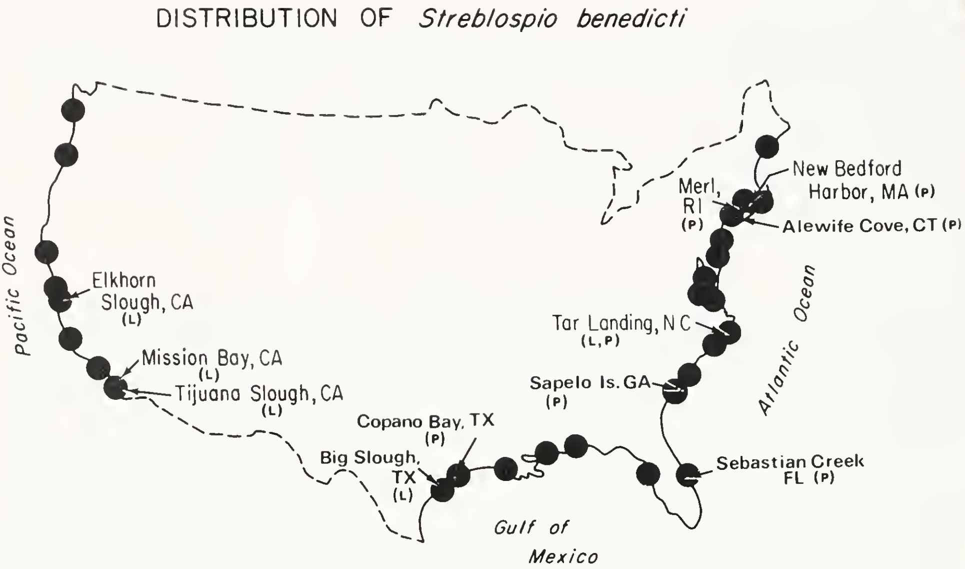

DISTRIBUTION OF *Streblospio benedicti*
Pacific Ocean
Elkhorn Slough, CA (L)
Mission Bay, CA (L)
Tijuana Slough, CA (L)
Copano Bay, TX (P)
Big Slough, TX (L)
Gulf of Mexico
Sebastian Creek FL (P)
Sapelo Is. GA (P)
Tar Landing, N C (L, P)
Atlantic Ocean
Merl, RI (P)
New Bedford Harbor, MA (P)
Alewife Cove, CT (P)

FIGURE 1. Distribution of *Streblospio benedicti* along the United States coastline, as given in the literature. Reproductive modes found at sampling sites in this study are labeled in parentheses ("L" = lecithotrophic, "P" = planktotrophic).

(8 cm diameter) containing fine sediments from Sippewissett Marsh in Falmouth, Massachusetts. Sediments (<1 mm diameter) were frozen (−30°C) to kill infauna, then mixed with sea water 5–7 days before addition to the polychaete cultures, to allow bacterial growth. The worms were given fresh sediment and mixed dinoflagellate cultures (*Prorocentrum, Perodinium, Gonyaulax, Gymnodinium*) every 4–5 days. Sea water and sediment were changed completely, approximately once a month.

Females brooding their young were isolated so that offspring from each parent could be followed separately. Duration of the planktonic period was determined by raising larvae individually in 13 × 100 mm glass test tubes at 20°C. The larvae were fed mixed dinoflagellate cultures, and sea water was not changed during the entire planktonic development. Fine sediment was added to the tube bottom in some vials to examine effects of substrate on development time or delay in settlement.

Reproductive features were measured on gravid females (relaxed with 1% $MgCl_2$) which had been freshly collected in the field or reared in the lab for 1–2 months. The characters quantified were: (1) body length (mm), (2) number of setigers, (3) maximum diameter of ova (μm), (4) number of ova per ovary, (5) first setiger involved in oogenesis, (6) number and position of brood pouches, (7) number of larvae brooded per pouch, and (8) brood size. Eggs in the coelom and larvae in brood pouches were observed through the transparent body wall of the female with a dissecting or compound microscope. Measurements were made using an ocular micrometer. In some instances early release of embryos in the female brood pouches was induced by prodding the (non-anesthetized) female. Embryos continued to develop normally, permitting the study of developmental stages which usually occur within brood structures. Larvae from populations in Mission Bay, Tijuana Slough, New Bedford Harbor, MERL, Big Slough, Copano Bay, and Sebastian River were observed and photographed at 1–2 day intervals following their release, using phase contrast optics (160×). The larval traits recorded were (1) size at release, (2) mode of nutrition, (3) setation, (4) swimming behavior, (5) length of planktonic period, and (6) size at settlement.

Preliminary crossing experiments were conducted to evaluate the ability of individuals from different populations to interbreed and to test for evidence of hermaphroditism, sex reversals, parthenogenesis, or other forms of asexual reproduction. Nine sex-blind crosses were made by placing pairs of immature juveniles from Mission Bay and New England (New Bedford and MERL) populations in separate culture dishes. In addition, reciprocal matings were performed between virgin or isolated females and males from Big Slough and MERL, Big Slough and New Bedford, Big Slough and Copano Bay, and Tar Landing and MERL.

RESULTS

Female reproductive traits

Oogenesis first occurs in setiger 7 but the exact segments involved varies among individuals and among populations. Oogenesis begins in more anterior segments in planktotrophic populations (setigers 7–11) than in lecithotrophic populations (setigers 12–14) (Table I). Ova develop within paired ovaries attached to genital blood vessels which extend into the coelomic space. The number of ova which develop in each segment also differs among individuals and among populations (Table I). In lecithotrophic populations 1–3 ($\bar{x} = 2.0$) ova may be observed through the body wall in each ovary. In planktotrophic populations 2–14 ($\bar{x} = 6.6$) ova develop in each ovary. The diameter of mature ova varies with trophic mode as well (Table I), ranging from 70 to 90 μm in planktotrophic populations and from 100 to 220 μm in lecithotrophic populations.

When ova are fully developed they move into posterior segments (presumably through the coelom), are fertilized by sperm stored in spermatophores, and enter coelomic brood pouches (Collier and Jones, 1967).

Larvae are brooded in paired dorso-lateral pouches (Fig. 2a) in all lecithotrophic and most planktotrophic populations observed (except those from Copano Bay and Sebastian River). Brood pouches can be present between setigers 17 and 45. They first appear between setigers 17 and 25 (Table I). The exact position varies with the size of the worm and, to some degree, among populations. Smaller worms have their brood pouches positioned more anteriorly than larger worms. The number of segments bearing pouches is positively correlated with the total number of setigers. For New Bedford $r = .67$, $P < .01$; for MERL $r = .87$, $P < .01$; for Tar Landing (Oct '83) $r = .65$, $P < .05$; and for Mission Bay $r = .83$, $P < .01$. Within each population larger worms, having more segments and more brood pouches, tend to produce greater numbers of larvae per brood than small individuals. Regressions of brood size on segment number are: for MERL $r = .72$, $P < .01$; for New Bedford $r = .57$, but $P > .05$; for Tar Landing in Oct. '83 $r = .70$, $P < .05$; for Big Slough $r = .38$, $P < .05$; and for Mission Bay $r = .42$, but $P > .05$. The slope of the regression line ranges from 6.7 to 7.9 in planktotrophic populations and from 0.71 to 2.60 in lecithotrophic populations.

The number of larvae brooded per pouch and the number of larvae produced per brood differ considerably in planktotrophic and lecithotrophic populations, independent of adult size (Table I, Fig. 3). Lecithotrophic females generally brood 1–2 larvae per pouch, in 5–16 paired pouches (Table I), to yield brood sizes of 8–59 (Fig. 3). Mean brood sizes for lecithotrophic populations are: 32 for Mission Bay ($n = 18$); 14 for Big Slough ($n = 28$); and 20 for Tar Landing Oct. '83 ($n = 9$). Over 95% of brood pouches bearing lecithotrophic embryos contain only 1 or 2 larvae

TABLE I

Female reproductive traits

	Planktotrophic populations					Lecithotrophic populations				Analysis of variance
	NEW BEDFORD HARBOR, MA	MERL, RI	TAR LANDING, NC 2/83	SEBASTIAN R., FL.	COPANO BAY, TX	MISSION BAY, CA	TIJUANA SLOUGH, CA	BIG SLOUGH, TX	TAR LANDING, NC 10/83	F
Total # of Segments (mature females)										
$\bar{x}$	54.8	43.4	56.0	69.0	44.5	49.8	49.4	44.1	41.3	$F_{8,191=36.39}^{*}$
S.D.	4.7	5.6	2.8	6.6	3.8	3.3	1.7	4.8	2.8	
n	52	48	5	3	6	30	8	39	11	
First Setiger Bearing Ova										
$\bar{x}$	11.5	10.2	11.1	11.3	7.8	13.5	14.0	12.6	12.1	$F_{8,187=20.13}^{*}$
S.D.	1.7	0.6	1.1	4.7	0.4	2.3	1.6	1.3	1.3	
n	46	50	7	4	6	33	10	31	9	
Ova/Ovary										
$\bar{x}$	6.3	5.3	8.0	7.0	6.3	2.0	2.1	1.9	1.9	$F_{8,166=33.78}^{*}$
S.D.	2.0	2.0	2.2	2.6	1.5	0.6	0.3	0.9	0.6	
n	35	53	6	4	6	25	9	29	8	
Ovum Diameter (all present)										
$\bar{x}$	69.7	59.1	62.9	68.0	56.7	115	152	140	152	$F_{8,168=21.89}^{*}$
S.D.	24.7	25.3	25.0	29.3	13.7	37	43	52	70	
n	39	54	7	4	6	28	8	22	9	
1st Setiger Bearing Brood Pouch										
$\bar{x}$	22.9	19.7	23.8	24.0	18.6	22.9	22.9	20.1	19.5	$F_{8,198=34.94}^{*}$
S.D.	1.6	1.5	1.1	2.6	0.5	1.0	1.2	1.3	1.9	
n	52	49	5	3	5	34	9	39	11	
Number of Brood Pouches										
$\bar{x}$	12.9	9.2	11.0	16.7	9.0	9.2	8.4	6.8	6.7	$F_{8,196=19.56}^{*}$
S.D.	3.3	2.7	1.3	1.5	2.7	2.0	1.5	1.8	1.8	
n	52	48	6	3	5	32	9	39	11	
Number of Larvae Per Pouch										
$\bar{x}$	8.7	6.6	8.0	—	—	2.7	2.0	1.9	1.9	$F_{6,90=51.72}^{*}$
S.D.	2.4	2.3	0	—	—	1.1	0.0	0.6	0.5	
n	13	19	3	—	—	18	2	31	11	
Brood Size										
$\bar{x}$	175.4	104.6	141.8	195.0	111.0	31.7	—	15.3	19.8	$F_{7,98=36.68}^{*}$
S.D.	47.2	59.0	45.2	113.1	31.6	10.7	—	7.5	10.6	
n	10	29	4	2	6	18	—	28	9	

* $P < .0001$.

(Fig. 2a, Table I). Occasionally lecithotrophic individuals collected in the field will brood up to three larvae per pouch, and one Mission Bay specimen reared in the laboratory was found with six lecithotrophic larvae in a single pouch. Planktotrophic females brood 4–14 larvae per pouch (Fig. 2b) in 5–23 paired pouches, releasing broods of 25–548 larvae (Fig. 3, Table I). Mean brood sizes are 175 (n = 10) for New Bedford; 105 (n = 26) for MERL (control tanks); and 142 (n = 5) for Tar Landing Feb. '83. A maximum brood size of 548 was observed for a *Streblospio* female collected from a MERL tank enriched at 8 × normal Narragansett Bay nutrient levels.

Planktotrophic development in females from Copano Bay and Sebastian River appears not to involve brood pouches. Embryos are brooded inside the parental tube against the dorsal surface of the female. They are partially enclosed by branchiae (vascularized lobes) present on segments between setigers 18 and 41 (Fig. 2c) and appear to adhere to the female. When she moves inside the tube the embryos are transported with her. The vascularized branchiae are similar in size and appearance to the large blood vessels embedded dorsally in the brood pouches present in other populations, and occur on those setigers which would otherwise be expected to support brood pouches. When the female leaves her tube, embryos or larvae are automatically released. Larvae brooded in pouches (in other populations) are normally retained within them, even outside the tube, unless the female is disturbed. Total brood sizes in these pouchless populations are comparable to those in pouch-brooding planktotrophic forms (Table I).

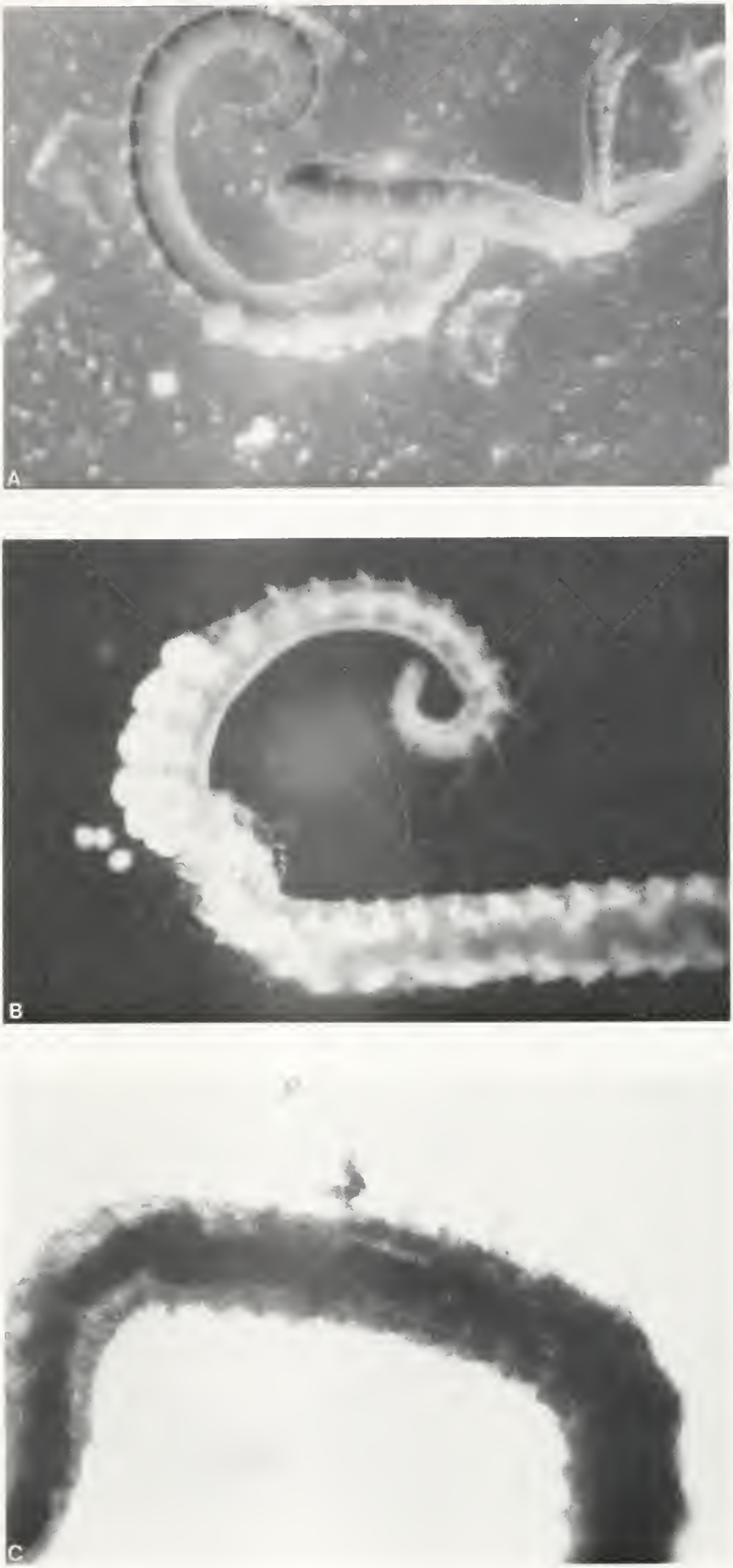
One Way Analysis of Variance of female reproductive traits yielded significant differences among populations for all characters tested (Table I). Student-Neuman-Keuls *a posteriori* tests documented distinct grouping of populations by trophic mode for the following traits: 1st ovigerous setiger, number of ova/ovary, ovum diameter, number of larvae per brood pouch, and brood size (Table II). In lecithotrophic populations oogenesis begins more posteriorly, produces fewer ova of larger diameter, and results in considerably smaller brood sizes than in planktotrophic populations.

Larval development

Lecithotrophic development was observed at all collection sites on the Pacific Coast, in the Gulf of Mexico at Big Slough, and on the Atlantic Coast at Tar Landing in October 1983. Planktotrophy was observed in specimens from all Atlantic Coast study sites including Tar Landing in February 1983 and from Copano Bay. Only in Tar Landing were both forms of larval development observed at a single site and these observations (of planktotrophy and lecithotrophy) were separated by ≈9 months. Later collections, made in early 1984, indicate that both planktotrophic and lecithotrophic forms are present at Tar Landing during January and February.

Laboratory cultures initiated from each collection bred true to the 'wild' type. Forms which were planktotrophic at the time of collection remained so and produced subsequent generations which were also planktotrophic. The same was true of lecithotrophic populations. Planktotrophic populations have been followed in the laboratory for up to 3 generations (≈3 mo/generation); and lecithotrophic populations for up to 4 generations (≈2–3 mo/generation).

Lecithotrophic development in *S. benedicti* is characterized by large ova, whose massive yolky embryos develop entirely within the brood pouch (Table III). Within 3 days of fertilization the trochophore is 300–340 μm in length. Segments are added at a rate of 1 or 2 per day and by day 7 or 8 (at 20°C) a fully developed larva (≈550–650 μm length) is released (Fig. 4A, B, and C). Late stage larvae may develop


A
B
C

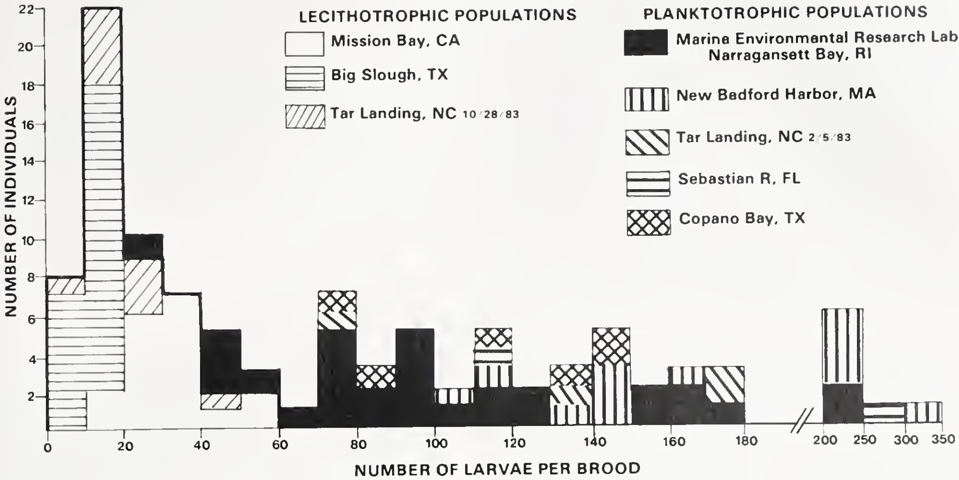


FIGURE 3. Observed distribution of brood sizes are shown in three lecithotrophic and five planktotrophic *Streblospio benedicti* populations.

adult setae (capillary setae and hooded hooks), but at no time do they exhibit the long swimming setae characteristic of many spionid larvae.

Lecithotrophic *S. benedicti* larvae are competent to settle at 9–12 setigers and generally settle at the time of their release. They may recruit without ever entering the water column. However, in the absence of suitable substrate, larvae may remain planktonic up to 7 days (Fig. 5). Following release from the brood pouch into the water column, larvae appear to derive nutrition from original yolk supplies. Stored yolk was observed to sustain larvae for approximately one week without external sources of food.

Planktotrophic *S. benedicti* larvae differ most noticeably from lecithotrophic forms in the presence of long "swimming" setae, a decreased yolk supply, a functional gut early in development, and a longer planktonic development (Table III). Within 2–3 days after fertilization (at 20°C), small ova develop into larvae with a single set of long serrated setae. Development usually proceeds inside the brood pouch to a 4-setiger stage within 24 hours and to 5–7 setigers within 2–3 more days (Fig. 4D, E, and F). Long serrated setae develop on each segment as they are added. The larvae have fully formed guts at the 4-setiger stage ($\approx$250–300 μm) and if released they begin planktonic feeding. Strong swimming ability and positive phototaxis is evident throughout the planktonic phase. Larval release normally takes place at 5–7 setigers (300–350 μm). Copano Bay and Sebastian River (pouchless) females have not been observed to brood beyond the 3 setiger stage. In other respects development of the pouched and pouchless planktotrophic forms appears to be identical.

Planktotrophic larvae settle within 2–3 weeks of their release at $\approx$9 setigers, though in the absence of suitable substrate the planktonic period may exceed 35 days

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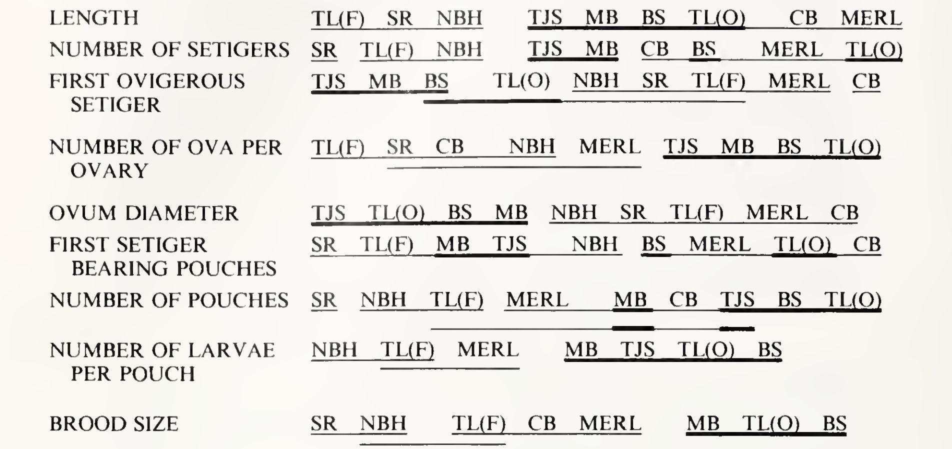
FIGURE 2. A. *S. benedicti* brood pouches bearing lecithotrophic embryos. Female collected from Big Slough, Texas, October, 1983. B. *S. benedicti* brood pouches bearing planktotrophic embryos. Female collected from New Bedford Harbor, Massachusetts, February, 1983. C. Brood structures on a *S. benedicti* female from Copano Bay, Texas, October, 1983. Note the absence of brood pouches. Vascularized branchiae appear in their place.

TABLE II

Reproductive similarities and differences among S. benedicti *populations*



LENGTH	TL(F) SR NBH TJS MB BS TL(O) CB MERL
NUMBER OF SETIGERS	SR TL(F) NBH TJS MB CB BS MERL TL(O)
FIRST OVIGEROUS SETIGER	TJS MB BS TL(O) NBH SR TL(F) MERL CB
NUMBER OF OVA PER OVARY	TL(F) SR CB NBH MERL TJS MB BS TL(O)
OVUM DIAMETER	TJS TL(O) BS MB NBH SR TL(F) MERL CB
FIRST SETIGER BEARING POUCHES	SR TL(F) MB TJS NBH BS MERL TL(O) CB
NUMBER OF POUCHES	SR NBH TL(F) MERL MB CB TJS BS TL(O)
NUMBER OF LARVAE PER POUCH	NBH TL(F) MERL MB TJS TL(O) BS
BROOD SIZE	SR NBH TL(F) CB MERL MB TL(O) BS

Abbreviations represent the following populations:

NBH = New Bedford Harbor, Massachusetts
MERL = Marine Experimental Research Laboratory, Narragansett Bay, Rhode Island
TL(O) = Tar Landing, North Carolina, October 1983 Collection
TL(F) = Tar Landing, North Carolina, February 1983 Collection
SR = Sebastian R, Florida
BS = Big Slough, Texas
CB = Copano Bay, Texas
TJS = Tijuana Slough, California
MB = Mission Bay, California

Results are based on Student-Neuman-Keuls *a posteriori* tests performed for each trait following One Way Analysis of Variance. Populations connected by lines are not significantly different. Heavy lines indicate lecithotrophic populations, light lines indicate planktotrophic populations. (See Table I for data.)

TABLE III

Contrasting patterns of larval development in Streblospio benedicti

	Planktotrophic Populations	Lecithotrophic Populations
# LARVAE BROODED/BROOD POUCH	4–14	1–2, occasionally 3
BROOD SIZE	$\bar{x}$ = 130, s = 65	$\bar{x}$ = 20, s = 12
DEVELOPMENT TIME TO RELEASE (20°C)	≈7 days	≈7 days
LARVAL STAGE AT RELEASE	3–7 setigers	9–12 setigers
LARVAL SIZE AT RELEASE	200–300 μm	500–650 μm
PLANKTONIC FEEDING	begins at 4 setigers	none
SWIMMING SETAE	present	reduced or absent
DURATION IN THE PLANKTON (20°C)	7–45 days	≤7 days
SIZE AT SETTLEMENT	450–550 μm	550–650 μm

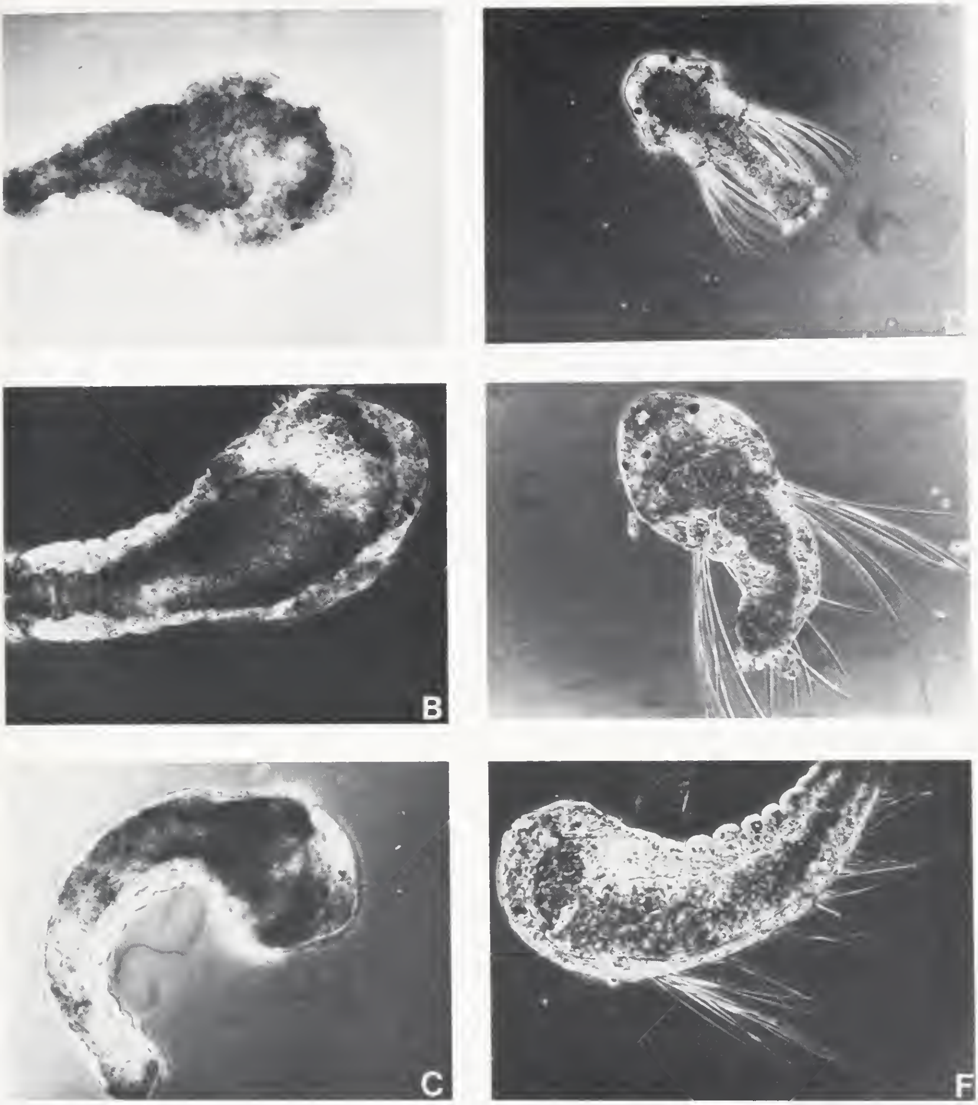


FIGURE 4. *Streblospio benedicti* larval stages. Note absence of swimming setae in lecithotrophic Pacific forms (A, B, C) and presence in planktotrophic Atlantic forms (D, E, F). A. Pacific Coast larva from a female collected in Tijuana Slough, California and cultured in the laboratory for one month. Shown four days after fertilization, 330 μm, four segments. B. Pacific Coast larva from the same brood (Tijuana Slough, California). Shown six days after fertilization, 535 μm, eight segments. C. Pacific Coast larva from the same brood (Tijuana Slough, California). Shown seven days after fertilization, 600 μm, nine segments. This is the stage at which release from the brood pouch normally occurs and at which the larva is competent to settle. D. Atlantic coast larva from a female collected in New Bedford Harbor, Massachusetts and cultured in the laboratory for one month. Shown three days after fertilization, 280 μm, one setiger. E. Atlantic coast larva from a female collected in MERL, Rhode Island. Shown five days after fertilization, 350 μm, five setigers. F. Atlantic coast larva from a female collected in New Bedford Harbor, Massachusetts. Shown 22 days after release, 500 μm, nine setigers.

(Fig. 5). The duration of the planktonic phase, particularly of the competent period (during which a larva is capable of settling), is highly variable in planktotrophic *S. benedicti,* even for larvae from a single brood. However, the probability of settlement

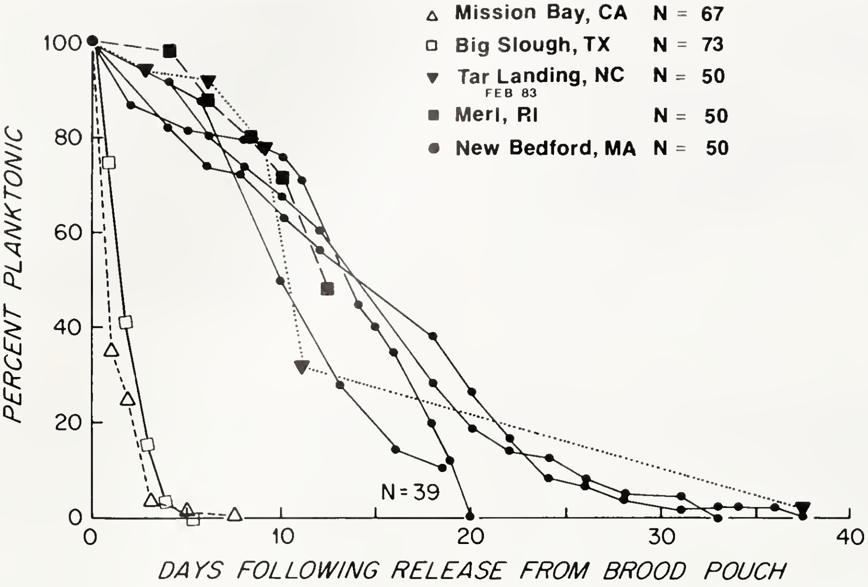


FIGURE 5. Planktonic period of *Streblospio benedicti* larvae raised individually in glass tubes without substrate at 20°C. Larvae were fed mixed phytoplankton cultures. Each line represents survivorship of individuals from a single brood or from pooled broods (Mission Bay, California, 3 broods and Big Slough, Texas, 7 broods). Loss from the plankton is taken to occur when larvae first permanently settle onto the tube bottom.

at any time after release is similar for larvae from different females and even from different populations (Fig. 5).

Interbreeding potential

Sex-blind pairings demonstrated that *S. benedicti* is a dioecious species which, at least under laboratory conditions, does not reproduce hermaphroditically or asexually. Analysis of nine pairings between Mission Bay juveniles and New England (MERL or New Bedford) juveniles after 2 months in culture revealed 4 male/female pairs, 4 female/female pairs, and 1 male/male pair. None of the single sex pairs showed any evidence of reproduction though all individuals contained ripe gametes. All male/female pairs had reproduced successfully, two producing lecithotrophic larvae by Mission Bay females and 2 producing planktotrophic larvae by New England females.

The F_1 larvae from crosses in both directions settled and successfully produced F_2 larvae by interbreeding among themselves. Additional inter-development mode reciprocal matings were conducted successfully between individuals from Big Slough × MERL, -× New Bedford, and -× Copano Bay, and between Tar Landing (lecithotrophs) × MERL. Results of these crosses will be presented in a later paper. They are mentioned here to demonstrate the interfertility of *S. benedicti* populations.

DISCUSSION

The observation of both planktotrophic and lecithotrophic development in *Streblospio benedicti* raises numerous mechanistic and evolutionary questions. The function of oogenic and vitellogenic processes, the relative roles of environment *versus* genotype, and the ecologic, taxonomic, and evolutionary consequences of the observed life history differences, have yet to be fully clarified.

Reproductive plasticity

The apparent plasticity of *S. benedicti* may be an evolutionary phenomenon in that different strains or subspecies may have differentiated with respect to mode of reproduction. An ecological alternative is that environmental cues trigger changes in oogenesis and vitellogenesis which produce the observed reproductive patterns. Eckelbarger (1980) reports that in *S. benedicti* there is a heterosynthetic uptake of blood pigment molecules from the blood vessel lumen in addition to autosynthetic vitellogenic processes. The ability to derive yolk precursors from extraovarian sources might allow *S. benedicti* to adjust ovum volume and number. Enrichment studies involving *S. benedicti* indicate that planktotrophic individuals can double or triple brood size in response to an increase in available organic matter (Levin, unpub.).

It is not known whether a single *S. benedicti* individual can switch reproductive modes and produce both planktotrophic and lecithotrophic larvae. This behavior was never observed in the laboratory. All 'wild' (field collected) specimens reared in the laboratory ($\approx$50 lines), and their progeny, exhibited reproductive characters identical to those of the field population. This observed continuity, and the occurrence of intermediate larval traits in F_2 progeny of F_1 hybrids resulting from inter-development mode crosses (Levin, in prep.), suggest that there is a strong genetic component to most of the reproductive characters monitored, including egg size, egg number, setation, and trophic mode.

The observation of both reproductive modes at Tar Landing (planktotrophy in Feb. '83 and lecithotrophy in Oct. '83) does suggest that individuals are capable of switching development mode. Alternatively, individuals with different genotypes within a single population may reproduce at different times, producing the observed pattern. The planktotrophic population sampled in February could have been replaced completely by recruitment of lecithotrophic individuals prior to the October sampling. The life span of *S. benedicti* individuals reared in the laboratory at 20°C (in the absence of predation) appears to be 6–12 months. However, generation times are only 2–3 months and individuals begin to lose reproductive vigor between 6 and 8 months of age.

The initial observation of dramatic life history differences among *Streblospio benedicti* populations suggested possible systematic differences between these forms. However, cross-breeding experiments demonstrate that the planktotrophic and lecithotrophic forms of *S. benedicti* are not reproductively isolated and thus do not merit species status. F_1 progeny resulting from crosses between the two forms are fertile as are subsequent generations (Levin, in prep.).

Ecological considerations

The reproductive variability of *S. benedicti* is probably partly responsible for the success of this species in North American bays and wetlands. This variation may have important implications for the population ecology of the species. Brood size, which can be ten or twenty times greater in planktotrophic than lecithotrophic *S. benedicti* (Fig. 3), will influence larval availability and potential rates of population increase (r). In both planktotrophic and lecithotrophic populations females produce multiple broods and larvae can rapidly colonize disturbed areas (Grassle and Grassle, 1974; Levin, in press; McCall, 1977; Oliver, pers. comm.; Quammen, 1981; Whitlatch, pers. comm.). However, the observed differences in larval planktonic period suggest that planktotrophic *S. benedicti* larvae possess much greater powers of dispersal. This conclusion is supported by the known occurrence of larvae in the plankton. In California bays, *S. benedicti* larvae, which are all lecithotrophic, are rarely collected in plankton tows. This was observed even when *S. benedicti* was the numerically dominant species in bottom sediments (Levin, in press; Blake, pers. comm; Nichols, pers. comm.). The reduced dispersal capability of lecithotrophic *S. benedicti* larvae is characteristic of many Pacific coast infaunal species (annelids, molluscs, and crustaceans) inhabiting back-bay environments (Levin, in press).

In contrast to the Pacific coast situation, planktotrophic *S. benedicti* larvae are often the most abundant component of the summer meroplankton in Atlantic coast estuaries (Dean, 1965; Simon and Brander, 1966). The ability of planktotrophic larvae to prolong planktonic development may improve chances of finding a suitable site for settlement under certain conditions. Planktotrophic *Streblospio,* in addition to a longer planktonic development, have stronger swimming abilities, faster speed, and more maneuverability (pers. obs. using videotapes) than lecithotrophic forms, and can feed in the water column during much of the development period. Thus, planktotrophic *S. benedicti* larvae appear to possess excellent dispersal abilities and probably exercise considerable powers of habitat selection.

In lecithotrophic forms, parental females may be more important than larvae in selection of offspring habitat. Studies in Mission Bay, California demonstrated that brooding females actively colonized disturbed sediments. Release of young followed by minimal dispersal resulted in rapid local increases in population size following colonization by only a few females (Levin, in press). Observations of high *S. benedicti* densities in artificial settling trays have led to the suggestion of *in situ* reproduction on the Atlantic coast as well (McCall, 1977; Whitlatch, pers. comm.) but it is not clear whether these studies involved planktotrophic or lecithotrophic forms.

I suggest from these findings that marine ecologists need to look carefully at the life history traits of community members. We should not assume that life history traits reported in the literature for a particular population will necessarily be accurate for different populations.

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TIME CUES FOR SEMILUNAR REPRODUCTION RHYTHMS IN EUROPEAN POPULATIONS OF *CLUNIO MARINUS*. II. THE INFLUENCE OF TIDAL TEMPERATURE CYCLES

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ABSTRACT

Breeding experiments with the intertidal midge *Clunio* demonstrate that tidal temperature cycles combined with the 24-h light-dark cycle can act as time cues controlling the semilunar (syn. lunar-semi-monthly) timing mechanism of the animals, thereby regulating the semilunar emergence rhythm of the population. The two environmental cycles are in identical phase relationships every 15 days. Two types of temperature cycles were examined, sinusoidal fluctuations and short term pulses of 1.5 h (3–5°C amplitude and 12.4 h period each). Comparing the entrained semilunar rhythms in terms of phase relationship to the time cues, the end of the warming interval seems to be the decisive parameter of the tidal temperature changes. The combined exposure to tidal temperature cycles and tidal cycles of mechanical disturbances of the water (an additional time cue in the populations examined) resulted in the correct semilunar synchronization when the temperature rises and the mechanical disturbances alternated as in a natural tidal cycle. The results from three stocks representing different geographical races are discussed in relation to the amplitude of tidal temperature changes in nature, to weak and strong influences of the time cues, to their perception, and to the phase relationship between rhythms and time cues in the experiments and in nature.

INTRODUCTION

The rise and fall of tides cause remarkable fluctuations in the physical environment of intertidal habitats. Among these fluctuations are the change from submergence to atmospheric exposure, the alterations of light intensity and of hydrostatic pressure, the mechanical disturbances correlated with stronger waves and breakers mainly during flooding, and, finally, the fluctuations of temperature. The variations between temperature conditions may be correlated with temperature differences between sea water and exposed substrates (depending on the time of day and the season), or between the higher and deeper layers of the rising and falling waters. However, intertidal organisms have evolved physiological properties that enable them (1) to tolerate these tidal fluctuations of environmental factors within the range of their habitat and (2), at least in many animal species, to time locomotor activity or reproduction by these factors relative to favorable intervals during exposure or inundation (for review see: Neumann, 1976c, 1981).

Populations of the intertidal chironomid *Clunio marinus* Hal. show a special adaptation to their habitat in the lower intertidal range. This adaptation is a semilunar (syn. lunar-semi-monthly) emergence rhythm which enables the immediately reproducing and short-lived midges to synchronize their egg deposition with the time of

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spring low water, at a certain time of day about every 15 days when the main parts of the larval habitat are generally exposed (Neumann, 1966). This semilunar emergence rhythm can be induced mechanically in the laboratory by artificial tidal cycles (period 12.4 h) of water disturbances if they are combined with a 24 h light-dark cycle (abbr. LD) such that both cycles have an identical phase relationship, about every 15 days, corresponding to the semilunar period of 14.76 days (Neumann, 1975; 1978). This, however, is valid only for the northern European populations of *Clunio marinus* (above 49°N latitude). The southern populations react to the monthly moonlight cycle which is an unreliable time cue (syn. zeitgeber) in northern latitudes (Neumann, 1966; Neumann and Heimbach, 1979).

Tidal changes in water temperature may also induce a semilunar emergence rhythm as suggested by preliminary experiments (Neumann, unpub.) and by observations of a sheltered *Clunio* population in a location with weak tidal water disturbances (Heimbach, 1976, 1978b). This paper demonstrates the zeitgeber effect of tidal cycles with temperature fluctuations on a semilunar reproduction rhythm.

MATERIAL AND METHODS

The stock "West Norway" used in this study originated from a population near Bergen/western Norway (60°16′N, 5°15′E), and was thoroughly described by Heimbach (1976, 1978b); the stock called "Dorset" came from Studland/southern England (50°39′N, 1°57′W) (Heimbach, 1976, 1978a); and the "Helgoland" stock originated from Helgoland Island in the German Bay/southern North Sea (54°11′N, 7°54′E) (Neumann, 1966, 1978). *Clunio* cultures were bred in air-conditioned rooms (Neumann, 1966). The light-dark cycles were LD 12:12 or LD 16:8. The light intensity for each culture bowl was constant during one experiment and ranged between 500 and 1500 lux, depending on the number of fluorescent tubes and their height above the cultures.

Two different types of artificial cyclic temperature fluctuations (period 12 h, 24 min, 50 s, cor. 12.4 h) were applied, namely "temperature pulses" and "temperature cycles." The experiments with *temperature pulses* were carried out in a tank, containing culture bowls, into which water was pumped from two basins, one dispensing warm water during a 15 min temperature rise, the other dispensing cold water during the rest of the tidal period (Fig. 1a, b). In this experiment great care was taken to avoid any tidal zeitgeber influence by mechanical disturbances (weak vibrations) which might result from the cooling system and the pumps. Control experiments showed that the entrained emergence rhythms were due only to water temperatures. In *temperature cycle* experiments only one basin was used in which the water was warmed over 6 h by a 70 W heating coil. The water was then allowed to cool in the air-conditioned room for the rest of the tidal period (Fig. 1c). A modified temperature cycle with a 15 min heater-induced *temperature rise* of 3–5°C followed by a slow temperature decrease was used in the Helgoland stock experiments (Fig. 8, 10) because some unknown aspect of the experimental conditions associated with the temperature cycles led to high mortality (3 trials).

Experiments testing the zeitgeber influence of artificial cyclic *mechanical water disturbances* were carried out in tanks subjected to vibrations of a synchronous motor (producing underwater sounds of 50–200 cps, 20–30 dB above background noise level). A program of 6 h vibration and 6.4 h silence simulated the tidal pattern of changing turbulence, bottom vibrations, and underwater sound in nature (Neumann, 1978).

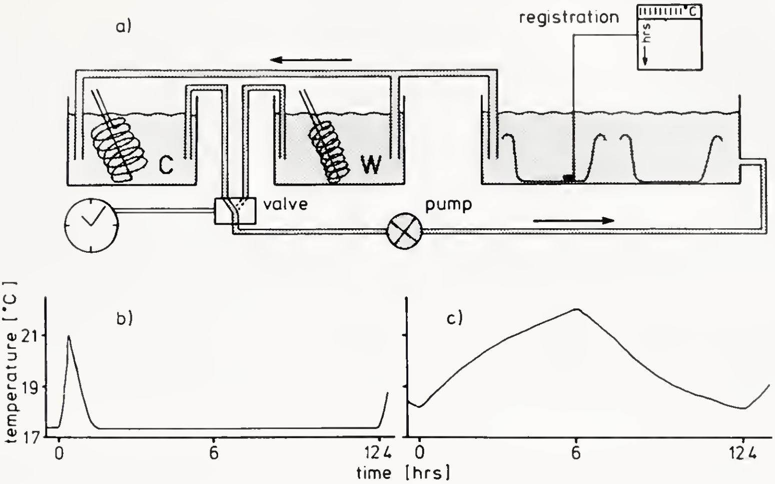

FIGURE 1. (a) Schematic sideview of the apparatus used for triggering a tidal temperature pulse within a tank with culture bowls (right side). C: Basin for cooling the circulating sea water to a standard temperature of about 18°C. W: Basin for warming the circulating sea water to 3–5°C above standard temperature. A valve controlled by a signal clock was switched every 12 h, 25 min to send the warm water into the basin for a period of 15 min. The water was later cooled to the standard temperature circulating through basin C. (b) Shows the resulting temperature cycle.

In the experiments with temperature cycles only one tank was used in which the water was warmed slowly every 12 h, 25 min for a period of 6 h. During the other 6 h, 25 min the water was cooled slowly by the lower room temperature. The resulting temperature cycle is shown in (c).

Temporal control of the artificial tides

The mean period of the semidiurnal tides is about 12.4 h (=12 h, 25 min), shifting daily by about 50 min. The local variations during the synodic month and the seasons depend on the moon's orbit, the influence of the sun's gravitation, and on the geographical characteristics of the sea basin as well as the sea coast. The mean semimonthly period of the syzygies (full and new moon, correlated at European coasts with spring tides) amounts to 14.76 days, half the synodic month of 29.53 days. Consequently, the spring tides recur about every 14th or 15th day. In spite of these odd periods and local irregularities, it is important to note that the phases of the spring tides (*e.g.*, high water and low water) occur on the average about the same time of day every year (*e.g.*, the mean time of day of spring low waters differs at Helgoland yearly by only a few minutes). To artificially simulate the tides and their semi-monthly recurrence at the same time of day, the semi-monthly period was rounded off to 15.0 days so that registration of emergence days and their evaluation was facilitated. The 29 artificial tidal cycles on these 15.0 days had a period of 12.413 h (=12 h, 24 min, 50 s) in order to reach equal phase relationships with the 24-h LD cycle every 15 days. Thus, each day of the semi-monthly zeitgeber cycle was characterized by a distinct phase relationship of the two zeitgeber factors (LD and tidal factor) (compare Fig. 2). The tidal cycles were triggered by a modified synchronous motor-driven 12-h clock. This clock was combined with two gears (29 and 30 teeth) so that the latter was rotating with a period of 12.413 h as described above (construction by F. Heimbach).

The temperatures in the *Clunio* habitats in Norway (Fig. 9) and Helgoland (Fig. 11) were recorded continuously by thermistors at different habitat locations. The tidal level was read from a marked vertical pole in the water.

RESULTS

When cultures of the *Clunio* stocks were exposed to tidal temperature cycles under strictly controlled laboratory conditions, a clear-cut semilunar emergence rhythm was induced within about 30 days resulting in emergence peaks every 15 days. This is seen in Figure 2 as distinct phase positions of the emergence peaks relative to the semi-monthly zeitgeber program *e.g.,* the tidal temperature cycles and the 24-h LD cycles. In control experiments lacking tidal temperature changes, and using the same environmental chambers, no semilunar emergence peaks were evoked (Fig. 3). Thus, the semilunar synchronization resulted exclusively from the semi-monthly pattern of tidal temperature cycles recurring on each of the 15 zeitgeber days that were in phase with the 24-h LD cycle. However, these experiments do not show which of the 15 zeitgeber days with phase-specific tidal temperature cycles was most effective in the synchronization processes. Nevertheless, the experiments do show that tidal temper-

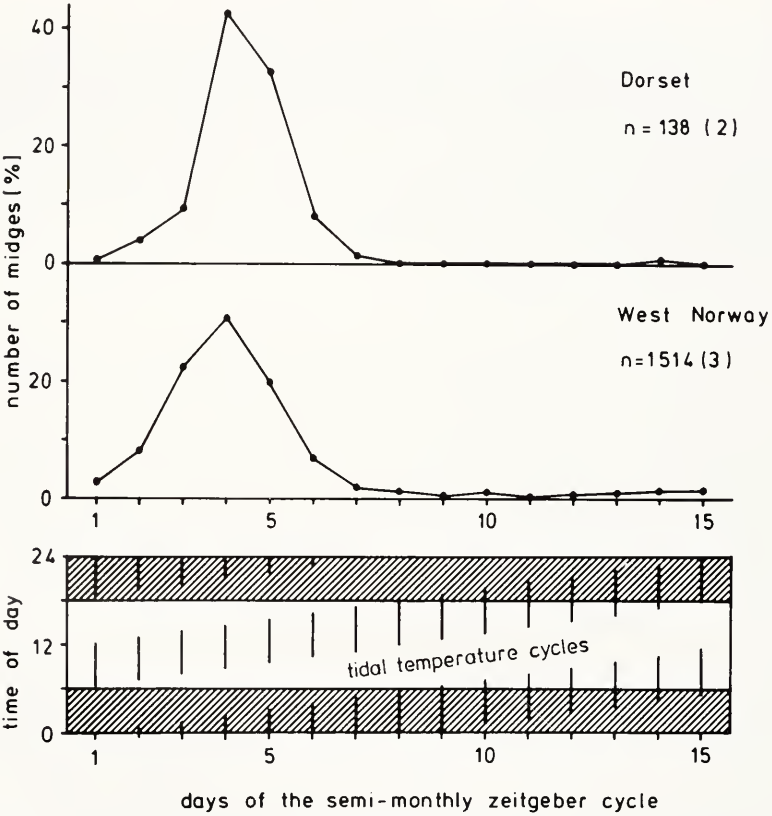

FIGURE 2. Semilunar emergence pattern of the *Clunio marinus* stocks in the LD 12:12, combined with a tidal temperature cycle of 12 h, 25 min duration (as in Fig. 1c) resulting in a semi-monthly zeitgeber program of recurring phase relationships. The upper graphs show the semilunar emergence rhythm of the two stocks, n = the number of midges emerged. The number of successive semi-monthly zeitgeber cycles evaluated (all with the same semilunar emergence pattern) is shown in brackets. The pretreatment by the same zeitgeber program consisted of 2 semi-monthly cycles each.

The graph at the bottom represents the daily warming periods (= vertical lines) during the semi-monthly zeitgeber cycle. Shaded area = darkness.

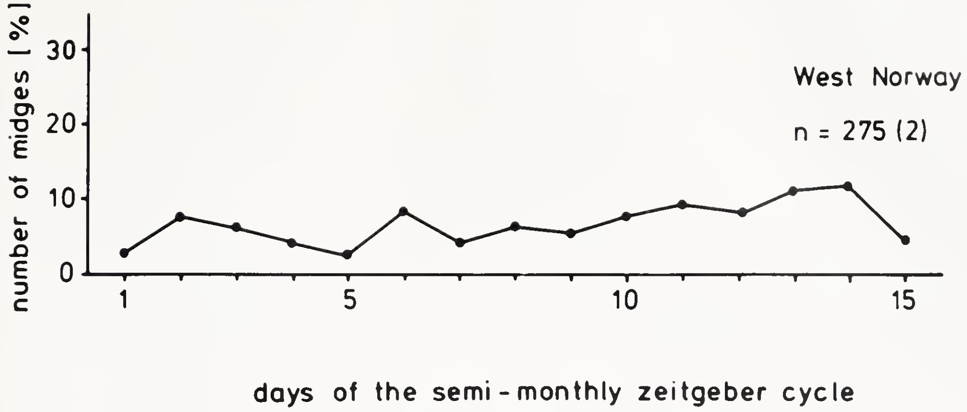

FIGURE 3. Control experiment for the experiments in Figure 2. Conditions: LD 12:12, but the tidal warming period was switched off in the breeding tank so that no tidal temperature fluctuations occurred. The mean temperature was ~18°C.

ature cycles with an amplitude of a few degrees Celsius may act together with the LD cycle as a zeitgeber for the semilunar-rhythmic emergence days.

Additional experiments evaluated the effect of short tidal temperature pulses (Fig. 1b) designed to simulate short temperature changes that may occur in nature during the time of low water. Even this modified program of tidal temperature fluctuations resulted in a precise semilunar-rhythmic emergence pattern with peaks every 15 days. These days corresponded to the semi-monthly zeitgeber program (Fig. 4).

A true semilunar-rhythmic synchronization of a *Clunio* population by environmental zeitgeber factors depends on an endogenous timing mechanism of the oscillatory "physiological clock" type (Neumann, 1966, 1976a, b). This timing mechanism autonomously oscillates with a period of about two weeks in each larva and permits the pupation in fullgrown larvae only during a few days every 15 days. This temporal control of the developmental processes results in a semilunar rhythm of emergence since the duration of pupation varies only within a small range of 3–5 days at temperatures between 15 and 23°C (Neumann, 1966; Krüger and Neumann, 1983). The effectiveness of this oscillatory timing mechanism is demonstrated when a synchronized population is transferred to conditions of 24-h LD cycles only (without any tidal or lunar zeitgeber conditions) and if a free-running circa-semilunar emergence rhythm of at least two peaks is registered. Such a free-run experiment was undertaken after a synchronization by tidal temperature cycles (Fig. 5). With two peaks at about 15 and 30 days after the last maximum controlled by the zeitgeber program it may be concluded that a circa-semilunar clock mechanism also is involved in these experiments with tidal temperature influences.

The phase relationship between an entrained circa-semilunar *Clunio* rhythm and its entraining semi-monthly zeitgeber program may be additionally influenced by the photoperiod, resulting in a delay of the emergence peak with increasing daylength as previously reported (Neumann and Heimbach, 1979). The same effect of the photoperiod was observed in an experiment with tidal temperature cycles where the delay of the emergence peak was about two days after a change from LD 12:12 conditions to LD 16:8 (compare Figs. 2 and 6, West Norway stock).

Cyclic mechanical disturbances of the water *e.g.*, turbulence, are additional factors which may act as zeitgeber on the entrainment of the semilunar emergence rhythm

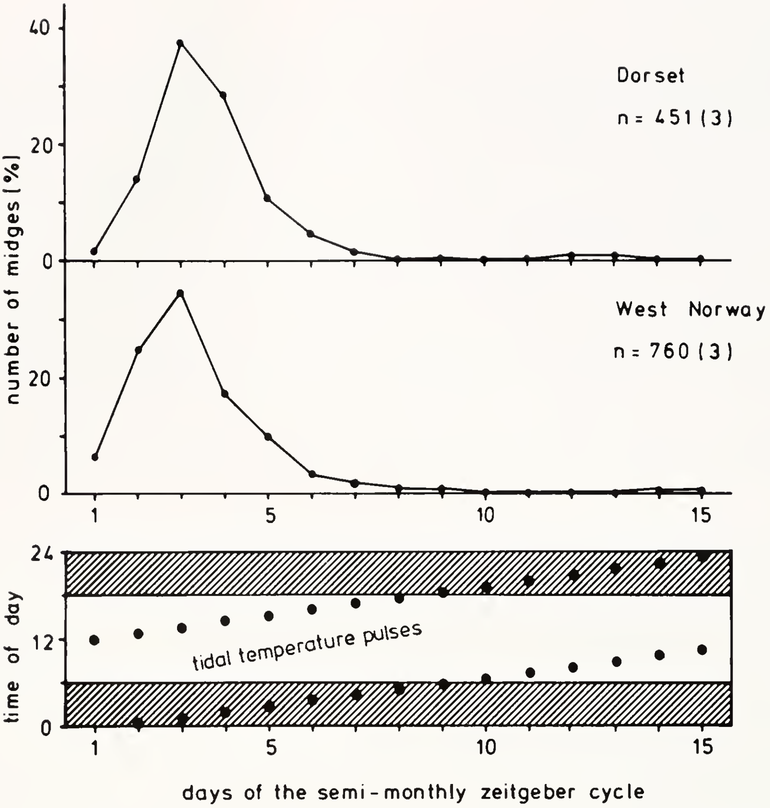

FIGURE 4. Semilunar emergence pattern of the two *Clunio* stocks in the LD 12:12, combined with temperature pulses of about 1.5 h length in regular intervals of 12 h, 25 min (cf. Fig. 1b). The dots in the bottom graph represent the time of day of the temperature pulses. See Figure 2 for further explanation. Data for the Dorset stock from Heimbach (1978a).

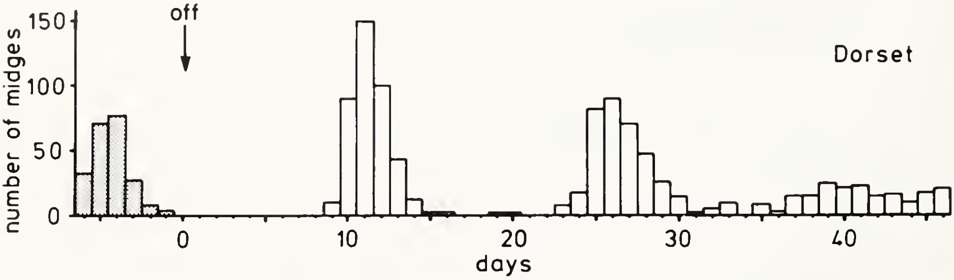

FIGURE 5. Free-running semilunar emergence rhythm of the *Clunio* stock from southern England in the LD 12:12, after entrainment of a semilunar emergence rhythm by the combination of LD 12:12 and tidal temperature pulses for several months as described in Figure 3. Dark columns: the last days of the experiment with temperature pulses; at day 0 the heating was switched off, while the other instruments (valve, pump, cooling, temperature control) remained switched on continuously.

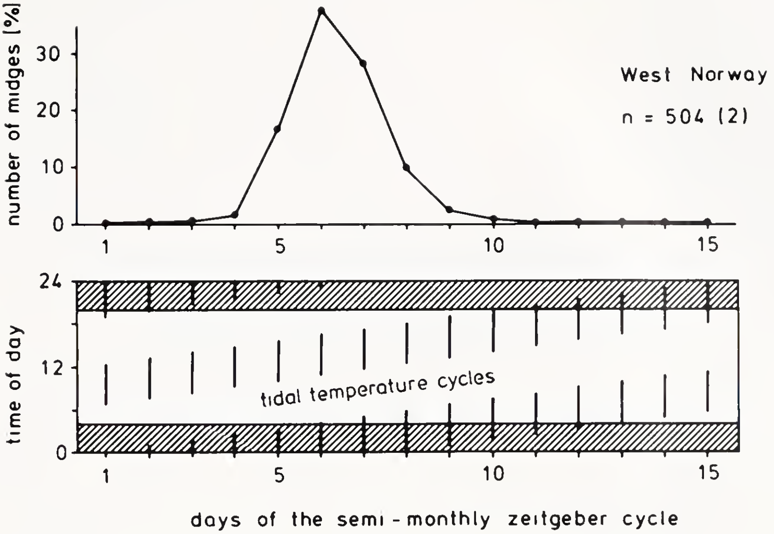

FIGURE 6. The zeitgeber effects of tidal temperature cycles (cf. Fig. 1c) combined with a LD 16:8, resulting in a semilunar emergence pattern in the West Norway stock of *Clunio marinus* (compare with Fig. 2). The temperature level in this experiment was about 3°C higher than in the experiments of Figure 2.

(Neumann, 1978). Three different aspects were considered in the following experiments: (a) strong or weak zeitgeber influence of this (turbulence) factor, (b) the phase relationship of the peaks within the semi-monthly zeitgeber cycle, and (c) the combined effectiveness of both time cues, the tidal cycles of temperature, and mechanical disturbances.

A relatively strong zeitgeber influence of the latter factor was established in the Dorset (Fig. 7, above; standard deviation S.D. = 1.9 days) and Helgoland (Fig. 8, above) stocks. The West Norway stock differed from such a precise synchronization, in that the mean semilunar-rhythmic distribution of the emerged midges was broader (Fig. 7, below; S.D. = 2.6 days). The standard deviation was even enlarged in an experiment with the same tidal program but with LD 12:12 instead of LD 16:8 conditions (no fig.; maximum value with only 14% midges on day 7, and values of about 10% on days 5–6 and 8–10, and relatively high values of 3–6% on the remaining days of the semi-monthly cycle; S.D. = 3.3 days). Mechanical disturbance of the water is apparently a weaker zeitgeber in the West Norway stock, whereas the temperature cycles have a stronger synchronizing effect (Figs. 1, 4). Conversely, the Helgoland and Dorset stocks respond to both zeitgeber factors with a clear-cut synchronization in longer and shorter photoperiods.

Such population-specific differences in the response to artificial zeitgeber conditions have been reported previously in *Clunio* stocks from north and south European coasts with respect to the influence of the monthly moonlight cycle and tidal water disturbances (Neumann, 1966, 1976a, 1978). These differences can be interpreted as the populations' genetic adaptations to reliable zeitgeber conditions of their geographical areas. The West Norway population represents a new type of physiological *Clunio* race that has a specific sensitivity to tidal temperature cycles. This adaptation of its circa-semilunar timing mechanism has ecological significance because clear-cut tem-

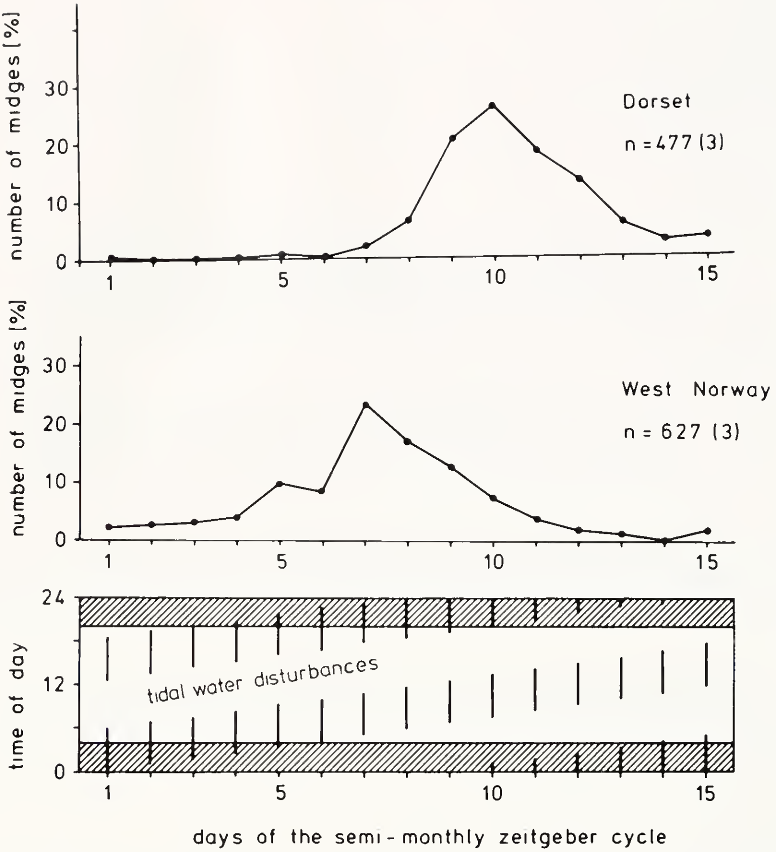

FIGURE 7. The influence of tidal mechanical water disturbances in a LD 16:8 on the emergence pattern of the two *Clunio marinus* stocks from southern England and western Norway. The vertical lines in the scheme below represent the time of daily water disturbances (6 hours every 12 h, 25 min) during the semi-monthly program.

perature fluctuations exist parallel with the tides (Fig. 9), while the changes of mechanical disturbances between ebb and flood are probably weak or often absent in the sheltered fjords with low amplitude tides.

If one compares the synchronization by the temperature zeitgeber with that by mechanical stimulation (Figs. 2, 7, Dorset stock; Fig. 8, Helgoland stock) one discovers differences of several days in the phase of the peaks relative to the semi-monthly zeitgeber cycle, even considering the Dorset experiment where a delay of two days was caused by the photoperiod influence described above. One may ask if these phase differences are merely a consequence of a randomly chosen correlation between both zeitgeber programs, or if they additionally demonstrate different properties of the physiological mechanisms for the perception of the temperature and mechanical zeitgebers. In fact, how both zeitgeber cycles can be exactly related to one another is a problem since in nature both factors reach their maximum values during different tide phases. The rise of temperature occurs during low tide and stops with the following

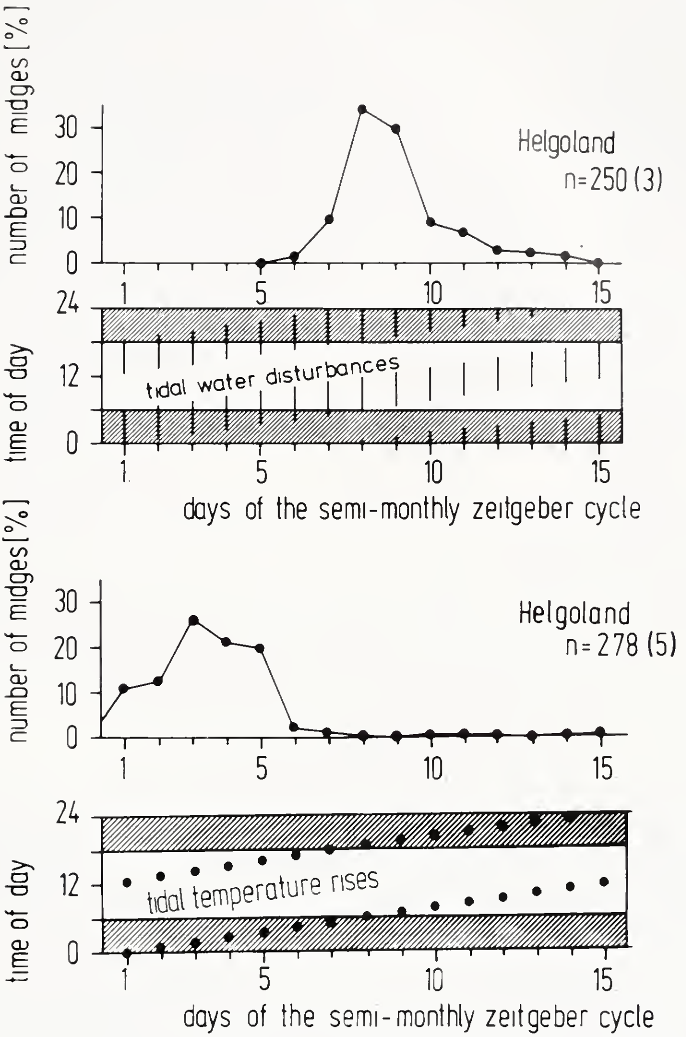

FIGURE 8. Helgoland stock of *Clunio marinus*. Mean semilunar emergence pattern entrained by tidal water disturbances (above) resp. tidal temperature rises (below) in LD 12:12. For comparison of both semi-monthly zeitgeber programs the data have been plotted in such a way that on each day the onset of the water disturbances (above) correlates with the end of the 15 min temperature rises (below).

inundation. The mechanical water disturbances are strongest during the rising flood. The comparison of the simplified laboratory zeitgeber programs with that occurring in nature as well as the correspondence of the phase relationship of the emergence peaks will be examined in more detail in the Discussion. However, in nature both tidal zeitgeber factors act during successive phases of the tidal cycle. Therefore the possibility of synchronizing the semilunar emergence rhythm with both tidal zeitgeber cycles simultaneously was tested in a final set of experiments (Fig. 10).

When mechanical disturbances and temperature increases alternated with each

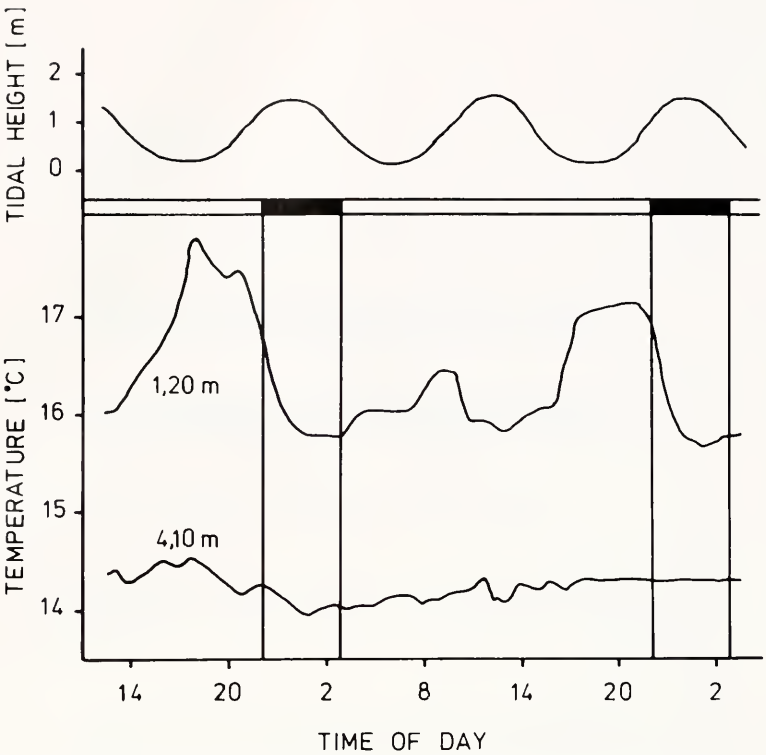

FIGURE 9. Temperature fluctuations in two habitats of different depths in Kviturdvikpollen near Bergen, western Norway in relation to tides and darkness (= black horizontal bar) on 10-8- and 11-8-76 (days of spring tide). The upper temperature curve was recorded at a depth of 1.2 m mean level (corresponding with the boundary between sub- and eulittoral), the lower one at a depth of 4.1 m. There had been sunny calm weather for several days. The maximum air temperatures at Bergen Airport were 21° and 18°C with wind forces 0–3.3 m/s.

other as in experiment 1 (Fig. 10), a normal semilunar emergence rhythm resulted with a peak of about 30% emerged midges at day 8 or 9 of the semi-monthly cycle, as was usual in this standard program with 6 h turbulence per tidal cycle (start of turbulence at midnight on day 1, 18–20°C LD 12:12). A specific reinforcement of the synchronization by the tidal temperature cycles could not be detected (compare Figs. 8 and 10). An irregular distribution of emerging midges resulted in experiment 4 (Fig. 10) when the tidal temperature increases always coincided with the start of the 6-h turbulence signal. In view of natural conditions in the intertidal zone, this was an unrealistic program because in nature the beginning of an inundation with increasing mechanical disturbances by breakers and a temperature rise at low water time differ by several hours. The experiment clearly demonstrates that in such a phase relationship of both tidal factors a mutual repression of their synchronizing effects must have occurred. The two other experiments with intermediate phase relationships of both factors (no. 2 and 3 in Fig. 10) resulted in a deviating entrainment of the semilunar emergence rhythm, with a small tendency for a delayed peak in number 2, and an even weaker synchronization in number 3.

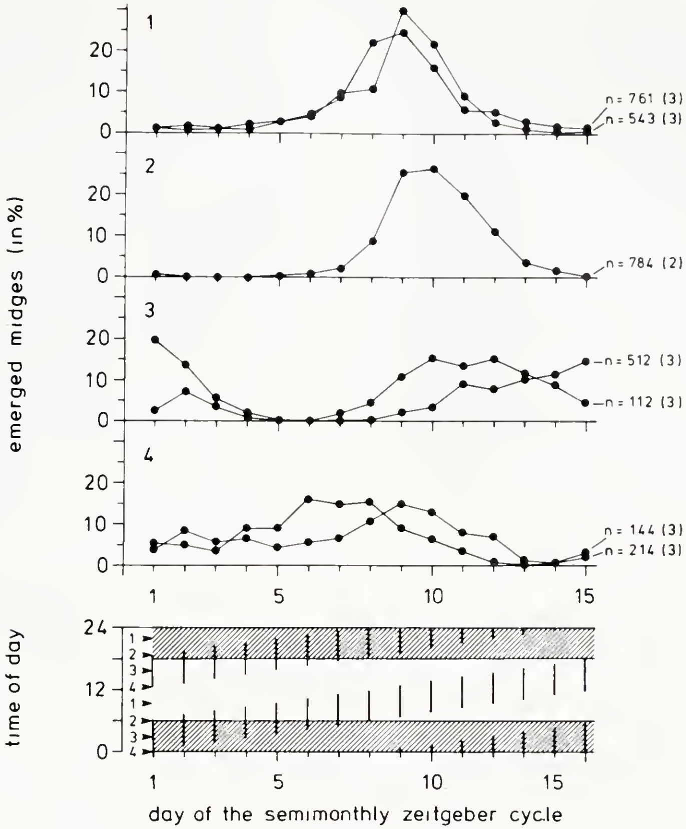

FIGURE 10. Helgoland stock of *Clunio marinus*. Influence of two combined tidal zeitgeber cycles on the semilunar emergence pattern. Both zeitgeber cycles—the tidal water disturbances and the tidal temperature rises—had identical phase relationships with the LD 12:12 every 15 days, but different phase relationships with each other, as shown in the lower graph. Experiment 1: temperature rise between successive tidal water disturbances (middle of simulated ebb); experiment 2: temperature rise at the end of each interval of water disturbances (start of simulated ebb); experiment 3: temperature rise during the middle of the water disturbance interval (midst of simulated flood); experiment 4: temperature rise at the onset of the water disturbance interval (start of simulated flood). The 4 upper graphs represent the mean distribution for emerged midges during the experimental program (n = number of emerged midges; numbers of semimonthly cycles in brackets). In experiments 1, 3, and 4 two independent series were conducted. Before evaluation of each experiment, the cultures were treated with the same zeitgeber program for at least 30 days. The lower graph represents the tidal water disturbances and standardized phase relationships with the LD conditions.

DISCUSSION

The foregoing experiments demonstrate that temperature fluctuations of 12.4-h period and of 3–5°C amplitude, in combination with a 24-h light-dark cycle, can act as an environmental time cue for the semilunar emergence rhythms of northern populations of the European *Clunio marinus*. These two environmental cycles (temperature and light) result in semi-monthly cycles of recurring phase relationships. Since neither of these periods (12.4 h and 24 h) contains the information for the 15 day period, one must conclude that only a distinct phase relationship of both during the semi-monthly program is effective for the entrainment of the biological rhythm. In the case of the zeitgeber influence of tidal cyclic water disturbances combined with daily light-dark cycles, this conclusion was strengthened by further experiments with modified zeitgeber conditions (Neumann, 1968, 1976b, 1978).

The amplitude of the tidal temperature changes

The influence of temperature change amplitude has not been examined in detail. However, the experimental changes (3–5°C) were within the range of natural conditions, as shown by field measurements. In the larval habitat area of the West Norway population of *C. marinus* (situated between the lower midlittoral and sublittoral of sheltered bays), a change in water temperature of up to 2°C was found parallel with the tidal rise and fall during a period of warm, calm weather (Fig. 9, upper curve). During the exposure of the habitat to air temperatures, the differences were probably even larger, due to higher air temperatures as well as to the solar radiation during daytime. At the *Clunio* location of Helgoland in April and May (spring swarming period), differences of up to 12°C have been registered between the exposed substrates at the time of low water and the mean water temperature (5–8°C). The changes were about 5°C on sunny days when mean water temperatures were 15–18°C (summer swarming period) (Krüger and Neumann, 1983). Similar temperature fluctuations may occur at the Dorset location (Heimbach, 1976). Temperature rises on the order of 2–12°C also have been measured in the upper sediment layers of a tidal mud flat (de Wilde and Berghuis, 1978).

The amplitude of tidal temperature changes will be modified by daily, lunar-semi-monthly, and seasonal variations. The daily variation occurs when the temperature increases mainly from direct solar radiation and fails to appear during night low water. This is valid for the Helgoland location (Fig. 11; compare also Krüger and Neumann, 1983). Lunar-semi-monthly variations of the mean water temperature (period 14.76 days) were found in a small sea area influenced by periodically drying mud flats where the semidiurnal lunar tide interacts with the daily radiation (Mok Bay at the Wadden Sea/Netherlands; Vugts and Zimmermann, 1975). The seasonal temperature variation and daily amplitudes follow seasonal weather conditions. However, the seasonal, as well as the lunar-semi-monthly modulations of the tidal tem-

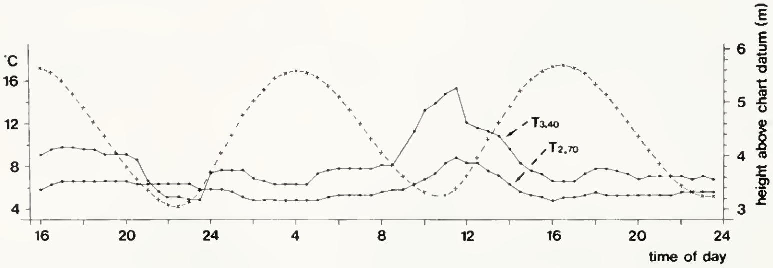

FIGURE 11. Daily variation of the tidal temperature fluctuation at Helgoland on 18 and 19 April, 1983. T-3.40: a thermistor which was located between *Clunio* substrates at 3.40 m height above chart level; it was exposed during low water time. T-2.70: a thermistor, in a water channel nearby, placed below the low water mark at 2.70 m. Air temperatures: 18 April: 3.3°C at 21:30 h, 19 April: 5.0°C at 14:30 h, 4.6 at 21:30 h, wind: SW-S, wind speed: 3.4–5.4 m/s, cloudiness: $^{1}/_{10}$ of sky at 14:30 h.

perature cycles, may be of little or no physiological significance for the entrainment of the semilunar rhythms of *Clunio* because minimum tidal changes of a few degrees Celsius occur on nearly all days during the swarming period (spring until autumn), and because lunar-semi-monthly temperature variations were not required for synchronization in the experiments. Finally, it is difficult to imagine how the insects could detect a lunar-semi-monthly fluctuation in mean daily temperature within the daily temperature fluctuations. Although no such experiments have been performed we suggest that the entrainment of the semilunar rhythm could be reliably triggered if only one of the two tidal temperature rises occurs per day.

Finally, the lower critical amplitude needs consideration. In an arctic *Clunio* population a tidal emergence rhythm was evoked by small temperature changes of only 1°C every 12.4 h (Pflüger, 1973). The circadian locomotory rhythm of the lizard *Lacerta sicula* was entrained in some specimens by a cyclic temperature difference of only 0.9°C (Hoffmann, 1968). Thus, one may expect that even tidal temperature increases of less than 3°C might be effective in the semilunar synchronization of the northern *Clunio* populations.

Perception of the tidal temperature rises

If only a distinct phase relationship between the tidal temperature rise and the day-night cycle is necessary for a semi-monthly zeitgeber program, then one of the physiological components of the *Clunio* timing mechanism for the semilunar control of pupation might be a circadian sensitive perception of the tidal temperature changes within a specific interval of the day. This hypothesis is similar to the zeitgeber perception model of the tidal cycles of mechanical water disturbances during the semi-monthly cycle (Neumann, 1976b). However, the physiological mechanism for perception of temperature changes must involve components other than those described in this model for two reasons: (1) the stimuli of mechanical disturbances were only effective when they lasted for at least 6 h within the 12.4 h period, in contrast to the effectiveness of the short tidal temperature rises; (2) from an ecological point of view, the sensitive circadian phase postulated should be different for both temperature and mechanical disturbance, because temperature increase during low water does not coincide with the effective change of the water disturbance from higher to lower values at the time of high water. This view agrees with the results of the experiment with combined tidal cycles of mechanical disturbance and temperature changes (p. 519).

The shape of the tidal temperature changes

The simulated tidal temperature fluctuations differed in its temporal shape: 15 min pulses and saw tooth curve (Fig. 1, p. 511). They can be characterized by different parameters: by the beginning and ending of the warming period, and by the steepness of the temperature increase and decrease. It is of both ecological and physiological interest to ask which of the parameters correlates with similar or even identical phase relationships of the entrained semilunar rhythms in the experiments. From the results in Table I the decisive parameter of the temperature zeitgeber was the end of the warming period (identical to the beginning of the temperature decrease). In nature this conclusion means that tidal temperature changes might occur at the end of low water when the exposed habitat in the lower midlittoral is again inundated. It is noteworthy that the steepness of the applied temperature increases was of no importance. These deductions should be relevant for any model of the tidal temperature perception within the physiological timing mechanisms of *Clunio*.

TABLE I

The phase relationship between the semilunar emergence rhythm of Clunio marinus *and the semi-monthly zeitgeber program in the experiments of Figures 2 and 4 as characterized by the time of day of the tidal warming on the days of maximum emergence*

	Tidal temp. cycles in Figure 2		Tidal temp. pulses in Figure 4	
Clunio stock	Start of warming	End of warming	Start of warming	End of warming
Dorset	20.15* 8.45	2.15 14.45	0.45 13.00	1.00 13.15
West Norway	20.15* 8.45	2.15 14.45	0.45 13.00	1.00 13.15

LD 12:12, 20°C.
The times of day are rounded off to quarters of an hour.
* Time of day on the evening before.

Comparison of experimental and natural phase relationships

To evaluate the quality of the entrainment of the semilunar rhythms of *Clunio* in the experiments, one should compare the artificial tidal conditions with the natural tides. One can confidently state that the temporal program of the artificial tides (either with temperature changes or mechanical disturbances) did simulate nature well because the peaks of the entrained rhythms recurred within each experiment on the same days as the arbitrarily defined semi-monthly zeitgeber cycle. Thus, it was possible, in each case, to plot the mean distribution of the emergence days as seen in the figures. However, with respect to the phase relationship between the rhythm and the tidal cycles, one must compare the natural and artificial conditions on a specific day, *e.g.*, the day of maximum emergence.

Detailed field observations on the emergence rhythm exist for the locations at Helgoland/North Sea during the summer swarming period. The peak emergence generally occurs on days of spring tides (one day after full and new moons; Caspers, 1951; Neumann, 1966; Neumann and Heimbach, 1979; Krüger and Neumann, 1983). At West Norway the semilunar-rhythmic maxima occurred under equivalent conditions, *i.e.*, days of spring tides about one day after the syzygies (Heimbach, 1976, 1978b). Comparable summer data for the Dorset/English Channel location are lacking. The only Dorset field data from the spring swarming period showed no obvious semilunar synchronization (Heimbach, 1976, 1978a). During April and May the semilunar emergence pattern of this location may be disturbed similar to that of the Helgoland location during spring when the habitat's temperature strongly fluctuates between cold water inundation and exposure to sunny warm weather (Krüger and Neumann, 1983). Thus, only the Helgoland and the West Norway data are given in Table II.

In relation to the chosen phase reference point, the onsets of the artificial tides of mechanical water disturbances differ from the natural tides and their low water time in the range of less than one hour (exactly with a $\Delta t_{B,C}$ of -30 min, Table II). This gives an excellent correlation between both the tidal pattern and the phase of the semilunar rhythm. This holds true even in experiments with a summer photoperiod of LD 16:8 where the emergence peak was delayed by about two days in the Helgoland

TABLE II

Tidal conditions characterized by the time of day of distinct reference points on days of maximum emergency

Tidal conditions	A[1] Temp. pulses or rises	B[2] Mech. disturbance	C[3] Natural tides	$\Delta t_{A,C}$	$\Delta t_{B,C}$
Tidal reference point	End of warming	Onset of mech. disturb.	Time of low water		
	Figure 8	Figure 8	Tide tables		
Helgoland	2.00	5.45	6.15		
	14.30	18.15	18.45	~4 h	<1 h
	Figure 4	Without Figure			
West Norway	1.00	5.00*	4.30		
	13.15	17.30*	17.00	~4 h	<1 h

[1] LD 12:12, ~20°C.
[2] Six hours duration, LD 12:12, ~20°C.
[3] Local time
* Weak entrainment of the semilunar rhythm.
Times of day rounded off to quarters of an hour, Δt values to whole hours.

stock and the $\Delta t_{B,C}$ resulted in approximately +1 h (Neumann and Heimbach, 1979). Although the entrainment was relatively weak in the West Norway stock, we suggest p. 516), we suggest that the tidal disturbances of the experiments act similarly to those in nature, as a stronger time cue for the Helgoland population, and as a weaker one for the West Norway population.

With regard to the tidal temperature pulses and the end of its warming period, there was a time difference of some hours compared to the natural tides ($\Delta t_{A,C}$ about 4 h in LD 12:12, Table II). In the summer photoperiod of LD 16:8 the emergence peak of the West Norway stock (Fig. 6) shifted to day 6 of the semimonthly cycle where the end of the warming interval was within the next range of the natural low water time (end of warming about 16.45 h, time of MLWS about 17.00 h). Thus, the experimental temperature cycles and the natural tides correlate well. However, in the LD 12:12 conditions there remains a discrepancy of several hours ($\Delta t_{A,C}$ in Table II) which was unexpected because the warming of the exposed habitat should end at about low water with the recurring flood. The abnormal shape of the temperature cycle (15 min temperature rise and slow cooling, p. 510) in the Helgoland experiments (Fig. 8, 10) may complicate a direct comparison of the 'end of heating' parameter with the low water time of natural tides. This tentative interpretation is consistent with the result of experiment 4 in Fig. 10 where the simultaneous zeitgeber effect of such a temperature rise and the onset of water disturbances was abolished. However, all of the experiments demonstrate that artificial tidal temperature cycles with an amplitude of only a few degrees can entrain a semilunar rhythm comparable to that seen in nature. Thus, we may conclude that the European *Clunio* populations have adapted to three different environmental time cues for the semilunar programming: monthly moonlight cycle, mechanical tidal water disturbances, and, as established in the present experiments, tidal temperature cycles. Each of these factors acts together with 24-h light-dark cycle.

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ARTIFICIAL PARTHENOGENESIS IN STARFISH EGGS: PRODUCTION OF PARTHENOGENETIC DEVELOPMENT THROUGH SUPPRESSION OF POLAR BODY FORMATION BY METHYLXANTHINES

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ABSTRACT

Methylxanthines such as caffeine, theophylline, and theobromine at 6 to 10 m*M* activated eggs of the starfish, *Asterina pectinifera.* Up to one hour of methylxanthine treatment induced parthenogenetic development in more than 80% of the eggs that failed to form the second polar body. Eggs that formed two polar bodies did not cleave. Compared with normally fertilized eggs the first cleavage in parthenogenetically developing eggs was delayed by 2 h in eggs lacking a polar body, and by 3 h in eggs with one (first) polar body. The intervals between successive cleavages were identical to those of normally fertilized eggs.

Examination of the chromosome number revealed that most parthenogenetic embryos develop as tetraploids. The possible significance of centriolar material in initiating parthenogenetic development is suggested.

INTRODUCTION

Since Oscar and Richard Hertwig's early success with artificial parthenogenesis (1887, 1896), sea urchin eggs have been the most common material used for experimentation on parthenogenetic development, while parthenogenesis in other marine ova has received much less attention. This is unfortunate because, among marine eggs, the sea urchin egg is unique in completing its meiosis before spawning.

Marine eggs that are fertilizable before completion of meiosis can undergo parthenogenesis. Thus, Tyler (1931, 1932), and Tyler and Bauer (1937) found that artificial parthenogenesis in *Urechis* is associated with suppression of polar body formation. Morris (1917) and Heilbrunn (1925) found that polar body formation and cleavage are mutually exclusive in artificially activated *Cumingia* eggs. Delage (1901) and Lillie (1908) noted that responsiveness of starfish oocytes to parthenogenetic agents declines as the oocyte goes through the first meiotic division. Apparently the formation of the polar body and parthenogenetic development are mutually exclusive.

Recently we found that caffeine and other methylxanthines are potent parthenogenetic agents in starfish eggs. Since methylxanthines inhibit cell division (Cheney, 1948; Roper, 1977; Stallwood and Davidson, 1977; Beetham and Tolmach, 1982), we used these agents to analyze the possible relationships between inhibition of meiosis and induction of parthenogenesis.

MATERIALS AND METHODS

Starfish, *Asterina pectinifera,* were collected during the breeding season and kept in aquaria with circulating cold (15–18°C) sea water until use. Immature oocytes

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free of the follicular envelope were prepared by treating isolated ovaries with Ca-free artificial sea water (CaFSW), as previously reported (Nemoto *et al.,* 1980). After several washings with normal sea water (NSW), we treated the isolated oocytes with 10^{-6} *M* 1-methyladenine (1-MeAde; Sigma Chemical Co.) in NSW to induce meiosis (Kanatani, 1969). Spermatozoa were obtained by making several cuts in isolated testes.

Methylxanthines such as caffeine (Sigma), theophylline (Wako Pure Chemical Co.), theobromine (Wako), and isobutyl-methylxanthine (Sigma) were dissolved in either NSW or CaFSW. These methylxanthines, at concentrations of 2–14 m*M,* were applied to eggs at various stages during meiosis. Technical details are described in Results.

To count chromosomes of the parthenogenetically developing embryos, the fertilization membrane was removed by washing with 1 *M* urea within a few minutes after the fertilization membrane began to elevate (Ikeda *et al.,* 1976). The denuded eggs were then transferred into either NSW or caffeine SW. The caffeine-treated eggs were returned to NSW 50 minutes later and allowed to develop. At the 16–32 cell stage eggs were treated with 3×10^{-4} *M* colchicine (Wako) in NSW for 10–15 minutes and then induced to swell by transferring them into diluted SW (30%). They were fixed for several hours at room temperature in a mixture of glacial acetic acid and absolute methanol in a 1:3 ratio and stored in 70% ethanol at −20°C. They were stained with aceto-orcein (Merck) for several days. Stained blastomeres were squashed on a slide glass and photographed or sketched to count the number of metaphase-arrested chromosomes.

RESULTS

Activation of maturing oocytes by methylxanthines

Caffeine was found to elevate the fertilization membrane in maturing oocytes. Figure 1A shows that 6 m*M* caffeine is the minimum concentration for formation

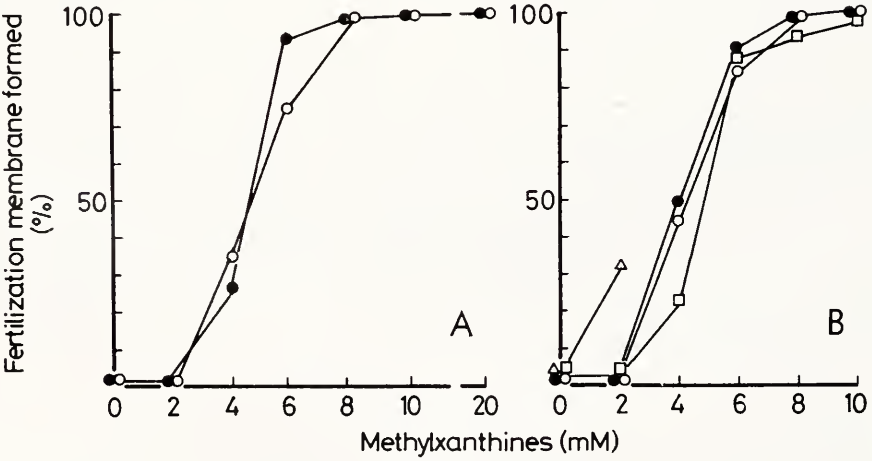

FIGURE 1. Methylxanthine concentrations required for the formation of a fertilization membrane (20°C). Each methylxanthine was applied to eggs between first and second polar body extrusion. (A) Caffeine in NSW (closed circle) and in CaFSW (open circle). Points are averages of 3 experiments. (B) Theophylline in NSW (closed circle) and in CaFSW (open circle); theobromine (square) and isobutyl-methylxanthine (triangle) in NSW. Points are averages of 2 experiments.

of the fertilization membrane in most eggs at any maturation stage after germinal vesicle breakdown (GVBD), just as the oocytes are fertilizable at any stage. The fertilization membrane became visible a few minutes after the application of caffeine, and elevated slowly taking several tens of minutes as in normally fertilized eggs. The formed membrane appears to be intact (Fig. 4A). Concurrently with the elevation of the fertilization membrane, oxygen consumption of the treated eggs increased transiently as in normal fertilization (Nemoto, Washitani, and Hino, in prep.). The term "activation" here means formation of the fertilization membrane by caffeine and other methylxanthines. The formation of the fertilization membrane following 10 m*M* caffeine treatment was inhibited by 4–6 m*M* procaine which suppresses the breakdown of the cortical granules of fertilized sea urchin eggs (Vacquier, 1975).

Other methylxanthines, such as theophylline and theobromine, had the same effects (Fig. 1B). Two m*M* isobutyl-methylxanthine also activated about 30% of the treated eggs. Owing to low solubility in NSW, however, the effects at higher concentrations could not be examined. *Asterias amurensis* and *Astropecten scoparius* eggs were also activated by these methylxanthines under similar concentrations (data not shown). Such effects of these methylxanthines were independent of Ca^{2+} in the external medium (Fig. 1A, B).

Duration of treatment with caffeine necessary to induce cleavage

The treatment with methylxanthines for a few minutes activated eggs as seen by elevation of a fertilization membrane; longer treatment allowed eggs to develop further if the eggs were returned to NSW after the treatment (see Figs. 4 and 5). In the experiments shown in Figure 2, caffeine (8 or 10 m*M*) was applied to eggs between first and second polar body formation. Fifty to ninety minute treatments were optimal

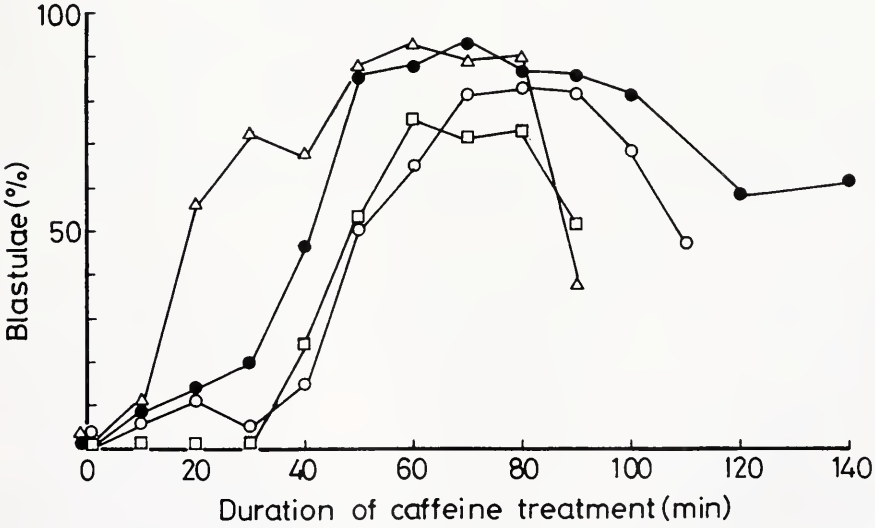

FIGURE 2. Duration of caffeine treatment required for parthenogenetic development (20°C). Caffeine at 8 m*M* (open circle) or 10 m*M* (other symbols) was applied to eggs between the first and second polar body extrusion. The number of developing embryos was scored (>500 eggs) about 10 hours after caffeine treatment. Points are averages for duplicate experiments.

(with some variation among batches) in obtaining a high frequency of blastula formation.

Cleavage and the timing of caffeine treatment during meiosis

The optimum period for insemination resulting in normal cleavage is known to lie between GVBD and the appearance of the first polar body although the starfish egg is fertilizable at any maturation stage after GVBD (Delage, 1901; Lillie, 1915; Fujimori and Hirai, 1979; Kominami and Satoh, 1980). We examined for such an optimum period in methylxanthine treatment by applying caffeine at various stages (Fig. 3A), *i.e.*, (I) within 5 min and (II) 15 min after GVBD, (III) within 5 min and (IV) 15 min after extrusion of the first polar body, and (V) within 5 min and (VI) 20 min after extrusion of the second polar body. The concentration of caffeine and duration of the treatment were 10 m*M* and 60 minutes respectively (Fig. 3B). Blastulae were produced when caffeine was applied before the appearance of the second polar body (treatments I–IV). High blastulae scores (78–84%) were obtained by treatments II, III, and IV. Conversely, very few eggs (less than 1%) cleaved when caffeine was introduced after extrusion of the second polar body (V, VI). Such a change in rate of development with delay of activation was noted by Delage (1901) in *Asterias glacialis* eggs and Lillie (1908) in *Asterias forbesii* eggs.

The majority of the eggs in treatment III started to divide about 3–4 hours after removal from caffeine SW to NSW (Fig. 4, A–D and Fig. 5, G–N). Once cleaved, the eggs usually continued to cleavage, reaching the blastula stage approximately 15 hours after the treatment and the early gastrula stage in another 20 hours. However, a large number of the eggs in I, V, and VI remained undivided, and eventually disintegrated (Fig. 4E, F).

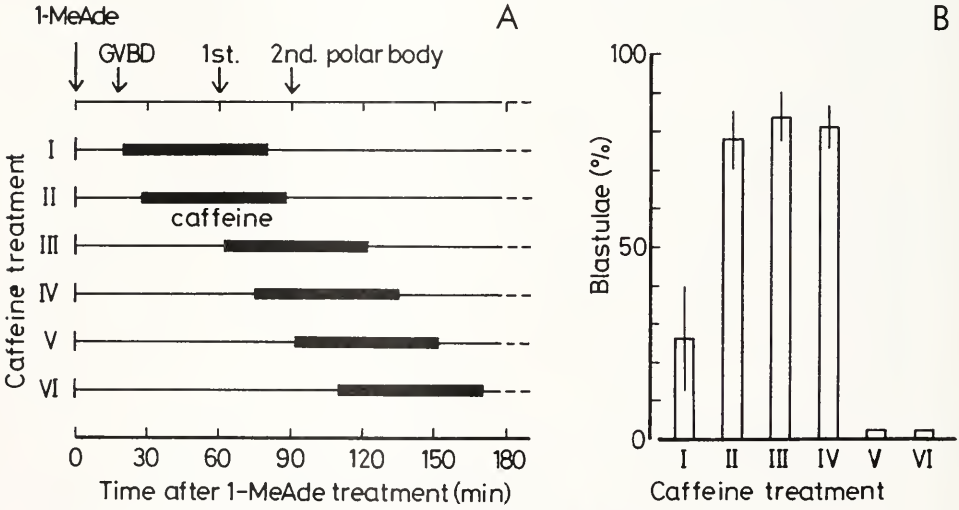

FIGURE. 3. Parthenogenesis of the eggs treated with 10 m*M* caffeine for 60 min at the various stages during meiosis (I–VI) as described in Results. 22°C. Time (measured after 1-MA) of the onset of GVBD (about 18 min), the first (60 min) and second (90 min) polar body is shown by arrows. Percentage of developing embryos (n > 500 eggs) 10 hours after caffeine treatment. Averages (columns) ±SEM of 4 experiments are shown.

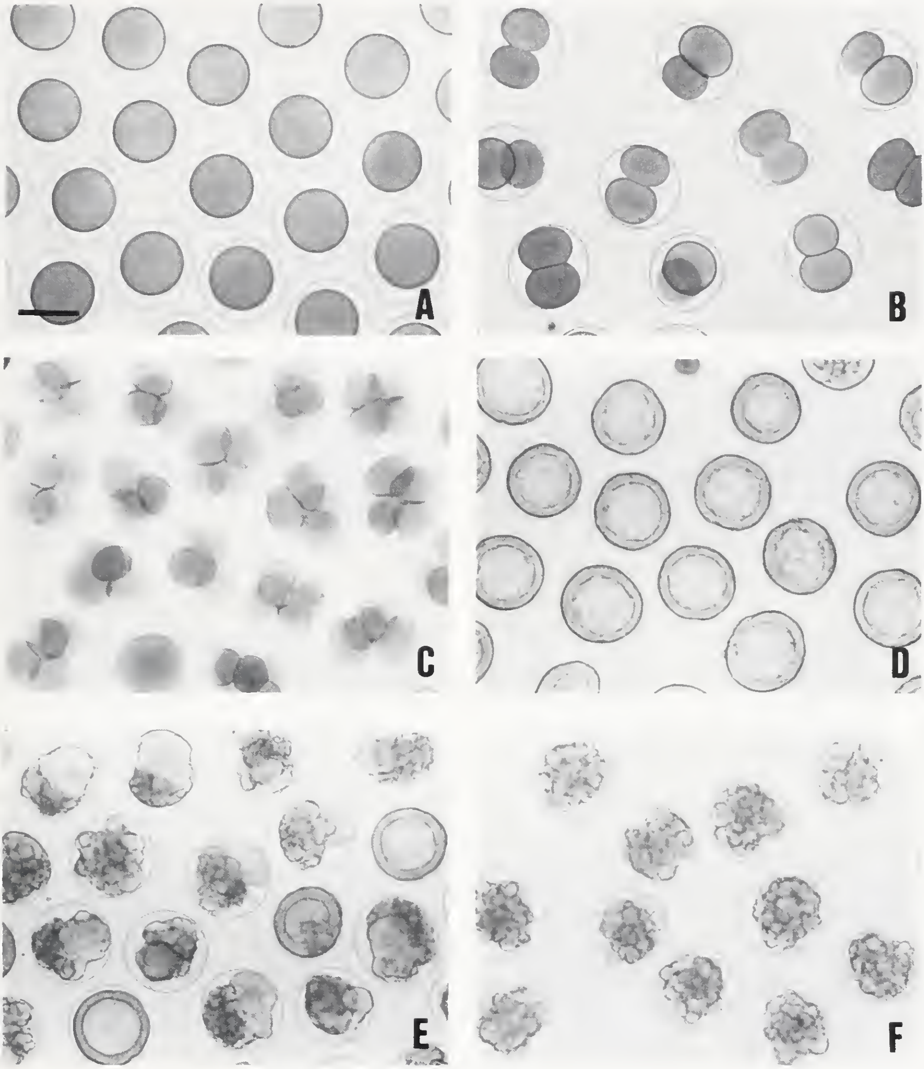

FIGURE 4. Development of eggs treated with caffeine (22°C). A–D: Eggs treated with caffeine shortly after extrusion of the first polar body; (A) 30 minutes after caffeine treatment, (B) 3.5 hours, (C) 4 hours, and (D) 16 hours. E–F: Disintegration of uncleaved eggs about 15 hours after caffeine treatment; (E) eggs treated just after GVBD (treatment I); and (F) eggs treated after extrusion of the second polar body (treatment V). Bar in A representing 100 μm is common to A through F.

While inducing parthenogenetic development, caffeine inhibited the formation of polar bodies in the treated eggs; in treatments III and IV, most eggs failed to extrude the second polar body. When subjected to treatment II, 90% of the eggs did not form any polar bodies. The remaining eggs formed only one polar body after they were returned to NSW, with a considerable delay in time. Eggs treated with caffeine in an earlier stage (treatment I) showed some variation (0–2) in the number of polar bodies formed. Moreover, two types of abnormal eggs were sometimes found; namely eggs with three polar bodies, and others with a single, distinctly large-sized polar body (Fig. 5, E–F) measuring 11.1 ± 0.6 μm (n = 16) in diameter. The first polar body in control eggs measured 8.2 ± 1.8 μm, n = 16. As in treatment II, polar body formation occurred after the treated eggs had been returned to NSW.

Caffeine and other methylxanthines used here inhibit cytokinesis in a variety of cells (Cheney, 1948; Roper, 1977; Stallwood and Davidson, 1977; Beetham and Tolmach, 1982). The present results show that caffeine also inhibits the meiotic division of starfish oocytes. Delage (1901) also reported that some parthenogenetic agents suppress polar body formation in *Asterias glacialis* oocytes.

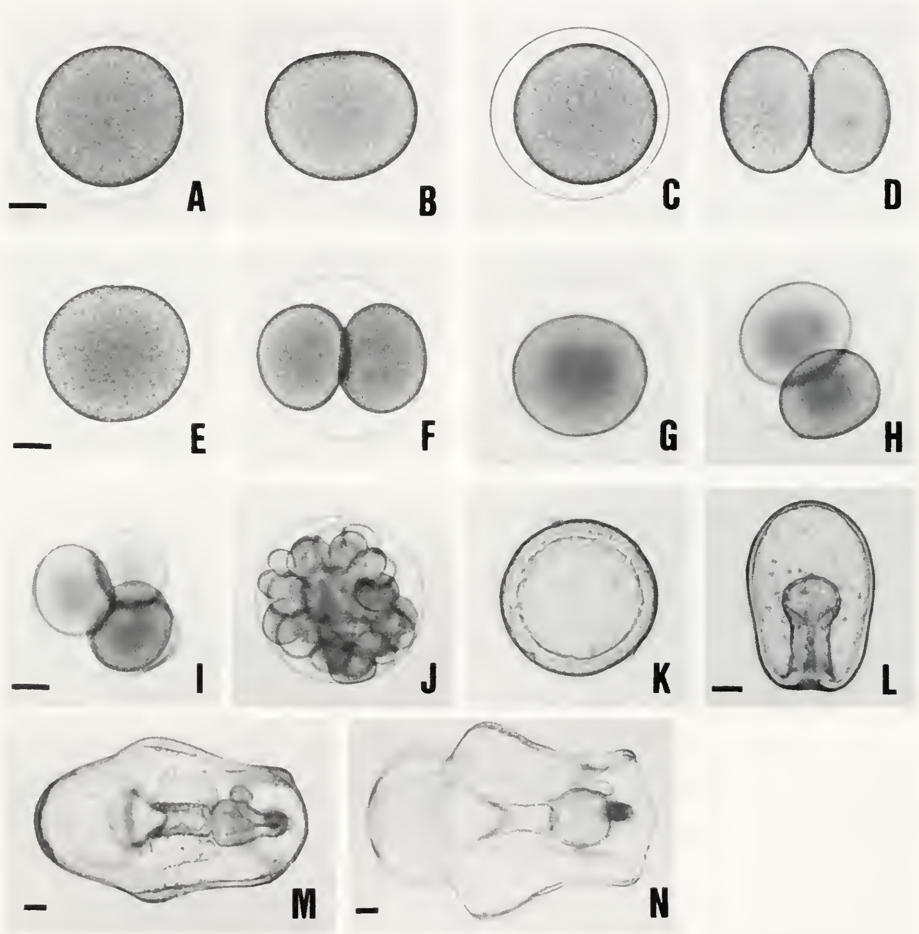

FIGURE 5. Cleavage of caffeine-treated eggs (22°C). A–B: Control eggs inseminated before first polar body extrusion; (A) 70 minutes and (B) 90 minutes after 1-MeAde treatment. C–D: Eggs with no polar body obtained by treatment II. E–F: Eggs with a giant polar body obtained by treatment I. G–N: Development of eggs with one polar body of normal size obtained from treatment III; (G) 2.5 hours after caffeine treatment (50 min), (H) 3.5 hours, (I) 4.0 hours, (J) 5.5 hours, (K) 16 hours, (L) 30 hours, (M) 3 days, and (N) 5 days. Bars represent 50 μm, and the scale in A is the same for B through K.

From the above it is clear that caffeine treatment can produce five kinds of eggs with respect to the number and size of the polar bodies: none, one, one giant, two, and three polar bodies. The results also suggest a correlation between capability of development and the number of polar bodies formed. To confirm this correlation, batches of eggs treated with caffeine at various meiotic stages were pooled and divided into the above 5 groups by inspecting individual eggs. Each egg was rolled around so that the polar bodies were not overlooked. The number of developing embryos was scored six hours later, and the number of polar bodies was again checked (Table I).

The eggs with either one normal-sized or no polar bodies divided with high frequency, whereas few eggs with either two or three polar bodies divided. The cleavage frequency among eggs with a giant polar body was about half that of eggs with a normal-sized polar body.

Caffeine as a parthenogenetic agent was found to induce, in some eggs, the formation of a giant polar body which was 2.4 times ($=[11.1/8.2]^3$) as large in volume as that of the controls. The appearance of such giant polar bodies also has been reported in artificially activated *Cumingia* (Morris, 1917; Heilbrunn, 1925) and *Urechis* eggs (Tyler, 1931). It is still unknown whether the formation of the giant polar body is causally related to parthenogenetic activation.

Cleavage timing

At the first cleavage, most caffeine-treated eggs divided into two cells (Fig. 5), although some eggs divided into three or four. Subsequent cleavages proceeded synchronously, and the cleaving eggs developed at least to the gastrula stage (Fig. 5, G–N).

After fertilization or caffeine treatment, individual eggs were transferred into separate wells of a culture plate. Direct observations were made every 5 min. An example of 5 experiments is shown in Table II. The first cleavage of caffeine-treated eggs was delayed as compared with that of control eggs. The time delay was about two hours in activated eggs with no polar body and about three hours in eggs with one normal-sized polar body. Hatching was also delayed corresponding to the delay of the first cleavage.

Synchronous cleavages of caffeine-treated eggs took place at 30 min intervals, which was nearly the same as that of control fertilized eggs. The time taken for hatching after the first cleavage did not differ significantly from that of the controls.

TABLE I

Number of polar bodies and rate of cleavage

Number of polar bodies formed	Eggs examined	Cleaved	Cleavage (%)
0	310	272	87
1 (normal)	356	317	89
1 (giant)	173	68	39
2	167	14	8
3	3	0	0

Five batches of eggs were used. Eggs of each batch were divded into four groups of caffeine treatments; I, II, III, and V (see Results). Five kinds of caffeine-treated eggs were selected before the first cleavage, and the rate of cleavage was counted 5–6 hours after caffeine treatment (10 m*M* and 60 min).

TABLE II

Timing of cleavages and hatching

	Time after 1-MeAde treatment (22°C)					
	1st	2nd	3rd	4th	5th	Hatch
(1)	4:00	4:20	4:50	5:40	6:20	16:00
Caffeine-treated eggs	4:15	4:45	5:15	5:40	6:00	16:35
with no polar bodies	4:05	4:35	5:05	5:30	5:55	15:40
	4:20	4:45	5:15	5:40	6:20	15:45
	4:05	4:30	5:05	5:25	6:00	16:10
	4:10	4:40	5:15	5:40	6:00	16:00
	4:10	4:40	5:15	5:40	6:05	15:30
	4:05	4:30	5:05	5:30	5:55	15:30
Mean	4:08	4:36	5:08	5:36	6:04	15:53
Interval		0:28	0:32	0:28	0:28	11:45*
(2)	5:10	5:20	6:05	6:30	7:05	17:05
Caffeine-treated eggs	5:20	5:45	6:05	6:30	7:15	17:15
with one polar body	5:15	5:45	6:10	6:35	7:05	16:45
(normal size)	5:05	5:30	6:05	6:30	7:05	16:55
	4:50	5:30	6:05	6:30	6:55	17:10
	5:30	5:55	6:20	6:45	7:15	17:35
	4:40	5:15	5:45	6:20	6:45	17:40
Mean	5:07	5:36	6:05	6:31	7:05	17:13
Interval		0:29	0:29	0:26	0:34	12:06*
(3)						
Control eggs						
Mean (n = 16)	2:14	2:42	3:10	3:38	4:10	13:59
Interval		0:28	0:32	0:28	0:28	11:45*

* Interval (hours and minutes) between the first cleavage and hatching.

Eggs were treated with caffeine (10 m*M* and 60 min) (1) after GVBD (treatment II) and (2) after extrusion of the first polar body (treatment IV). Control eggs were fertilized before extrusion of the first polar body.

Prior to the first cleavage, multiple nuclei appeared in the caffeine-treated eggs. Subsequently they fused, and then disappeared to enter mitosis. In *A. glacialis,* Tsukahara and Ishikawa (1980) have described the appearance of multiple nuclei when polar body formation was suppressed by cytochalasin B, and their fusion after thymol activation.

Number of chromosomes in normal and parthenogenetic embryos

Makino and Niiyama (1947), counted 40 chromosomes (diploid) in sectioned preparations of male *A. pectinifera* gonads. In our experiments, 44 (2n) chromosomes were found in normal embryos, although a slight variation was noticed (Fig. 6). In caffeine-induced embryos the number of chromosomes ranged from 78 to 107, with a median of 88 (Fig. 7). No significant difference in chromosome number was found

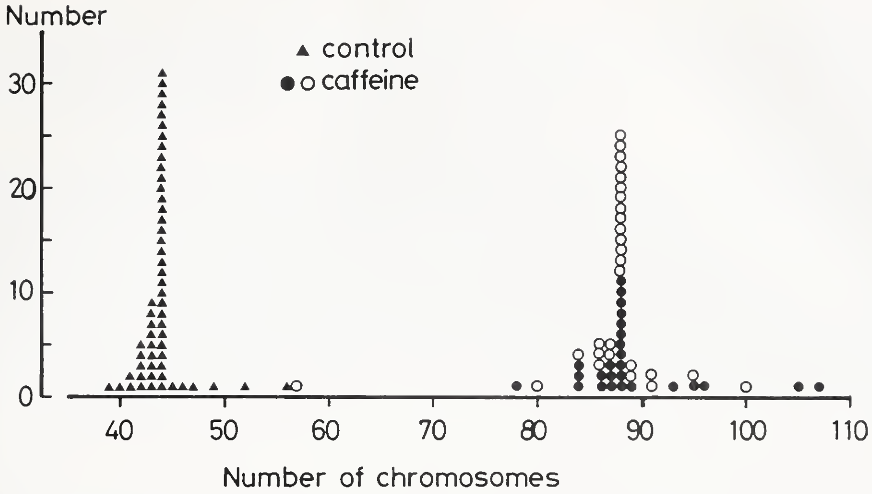

FIGURE 6. Histogram of chromosome number. Most control blastomeres had 44 chromosomes (triangle). A peak of chromosome number in caffeine-induced embryos with one polar body (open circle) and with no polar bodies (closed circle) was 88. Mosaic of chromosome numbers in individual parthenogenetic embryos was not found.

between parthenogenetic embryos with no polar body (closed circle) and one polar body of normal size (open circle). Obviously parthenogenetic embryos are tetraploids.

Since the intervals between successive cleavages are identical to those of normal eggs as described above, these caffeine-induced embryos must have become tetraploids before the first cleavage. The tetraploid status is maintained at least up to the early gastrula stage (Mita and Obata, unpub. data).

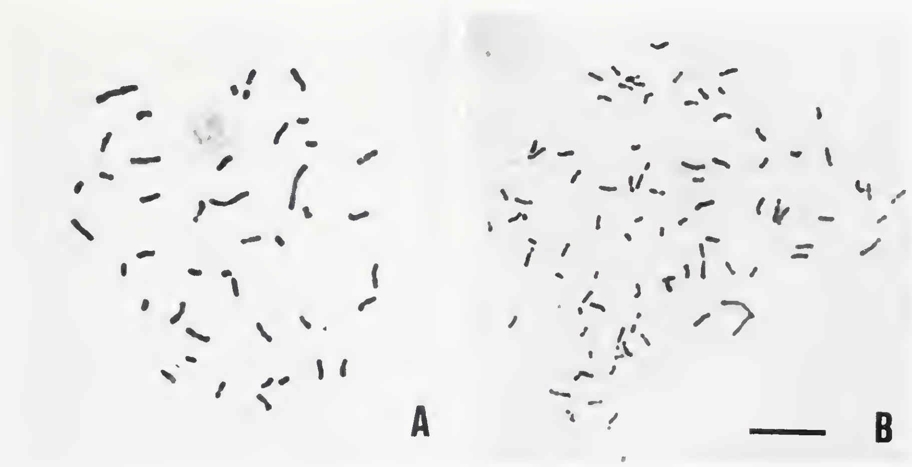

FIGURE 7. Chromosomes of a normal embryo (A) and a caffeine-produced embryo (B). Bar: 10 μm. Chromosome numbers are 44 (2n) and 88 (4n) respectively.

DISCUSSION

The action of methylxanthines on starfish eggs as a parthenogenetic agent may occur in two steps, *i.e.*, the activation of the eggs and the initiation of parthenogenetic development.

An increase in intracellular free calcium is an immediate consequence of fertilization or activation in sea urchin eggs, and sea urchin eggs are activated by those agents that increase intracellular Ca^{+2} (Nakamura and Yasumasu, 1974; Steinhardt *et al.*, 1974, 1977; Vacquier, 1975; Zucker *et al.*, 1978; Hamaguchi and Hiramoto, 1981). Caffeine reportedly releases Ca^{2+} from the sarcoplasmic reticulum of muscle tissues (Weber and Herz, 1968; Endo *et al.*, 1970). Caffeine and the other methylxanthines used here, like Ca^{2+}-ionophores, shorten the length of the "1-MeAde-requiring period" in meiosis reinitiation of starfish oocytes (Nemoto, 1982). Therefore, methylxanthines may cause activation by an increase in intracellular Ca^{2+}. Since these methylxanthines acted independently of calcium in the external medium, Ca^{+2} may be used from some intracellular storage.

Suppression of polar body formation by methylxanthines may be due to a disruptive effect on the meiotic spindle. Harris (1983) recently observed that caffeine prevents normal separation and function of the mitotic centers in fertilized sea urchin eggs, resulting in periodic monoaster formation rather than division. As a result, caffeine inhibits separation of daughter nuclei. In fact we found that the multiple nuclei formed by caffeine treatment subsequently fused.

How such a change in nuclear behavior produces parthenogenetic development is unclear. Possibly the duplication of chromosomes by the fusion of once separated daughter nuclei triggers parthenogenetic development. However, this possibility does not explain the observed chromosome number of parthenogenetic embryos; the fused nuclei of the egg without forming the polar body would be tetraploid, whereas the nuclei of those eggs that formed one polar body would be diploid. The observed embryos derived from both types of eggs all develop as tetraploids. Since the interval between the first and fifth cleavage in caffeine-treated eggs was almost the same as that of the controls, these caffeine-induced embryos must have become tetraploid before the first cleavage. An extra delay of the first cleavage by one hour in eggs with one polar body (Table II) may be the time taken for an extra round of DNA synthesis. Von Ledebur-Villiger (1972) found that the majority of sea urchin embryos developing parthenogenetically from ripe haploid eggs are still tetraploids. How most parthenogenetic embryos develop as tetraploids is still unknown.

The role of centrioles has been emphasized in the development of sea urchin eggs. It is believed that in normally fertilized sea urchin eggs centrioles derived from the sperm act as the mitotic organizing center of the zygote nucleus, since ripe unfertilized eggs lack centrioles (Sachs and Anderson, 1970). Therefore, artificial parthenogenesis in sea urchin eggs has been assumed to involve "*de novo*" formation of centrioles (Sachs and Anderson, 1970; Kuriyama and Borisy, 1983). However, it should be recalled that the actual number of developing eggs is unusually low; only 1–3% (rarely up to 30–40%) of artificially activated eggs develop into blastulae or plutei (Von Ledebur-Villiger, 1972). By contrast, in the present study (*cf.* Fig. 3 and Table I) more than 85% of the starfish oocytes developed to blastulae if treated before formation of the second polar body, whereas only a few percent of mature eggs (two polar bodies) were induced to develop. Since the maturing oocytes should retain at least one set of centrioles for organizing centers of meiotic division, it is possible that the centrioles can be diverted into an organizing center of mitotic division when the oocytes are activated by caffeine. This would explain the observed high frequency of parthenogenetic development of maturing oocytes in contrast to the very low rate in mature eggs of starfish and sea urchins.

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RELATIONSHIP BETWEEN GROWTH, DIFFERENTIATION, AND LENGTH OF LARVAL LIFE FOR INDIVIDUALLY REARED LARVAE OF THE MARINE GASTROPOD, *CREPIDULA FORNICATA*

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ABSTRACT

Larvae of the gastropod *Crepidula fornicata* were reared individually through spontaneous metamorphosis in clean glass containers at constant temperatures ranging from 15°C to 29°C; each larva was examined daily. Growth rates were determined from periodic measurements of individual shell length. Differentiation rates were estimated as (days to development of gill rudiments)$^{-1}$ and as (days until shift from larval to adult shell geometry)$^{-1}$. Growth ceased abruptly in a majority of the larvae in each treatment, over the size range 900–1100 μm shell length. Larvae continued to ingest phytoplankton during this period, and growth resumed at a normal rate following spontaneous metamorphosis. An inverse correlation was observed between rates of larval growth and length of larval life through spontaneous metamorphosis; faster-growing larvae generally had shorter larval lives than did slower growing larvae. Individual growth rate (μm/day) prior to competence was significantly correlated with rate of individual differentiation. However, rates of differentiation and growth as measured in this study were comparable predictors of when spontaneous metamorphosis would occur. The results are consistent with the hypothesis of a pre-programmed end to larval life in the planktotrophic larvae of *C. fornicata,* although the factors responsible for initiation of gill development and the shift in shell morphology are apparently not directly related to progress towards the point at which the larva spontaneously metamorphoses to the benthos.

INTRODUCTION

Metamorphosis of many marine invertebrate larvae involves the loss of a specialized swimming organ or structure, and the consequent exchange of a free-living, planktonic life for a relatively sedentary, benthic one. Larvae of most marine benthic invertebrates are able to postpone this time of morphological and ecological transition in the absence of environmental cues characteristic of the appropriate adult habitat (Crisp, 1974; Scheltema, 1974). Species differ significantly in the length of time that metamorphosis can be delayed, and thus differ in maximum dispersal capability (Pechenik, 1980; Jackson and Strathmann, 1981). The length of time that metamorphosis can be delayed often appears to be related to rate of larval development (Pechenik, 1980, 1984; Jackson and Strathmann, 1981), even for species with feeding (planktotrophic) larvae. In particular, a significant inverse correlation between rate of growth and maximum duration of planktonic life has been documented for larvae of the prosobranch gastropod *Crepidula fornicata* (Pechenik, 1984). This relationship is observed for larvae within a single culture, and among cultures held at different temperatures.

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The relationship is also apparent for larvae of *C. fornicata* reared at different food concentrations at a single temperature (unpub. data). An end to larval life appears to be somehow developmentally pre-determined for these species; the duration of planktonic existence may thus be limited by the rate at which individuals progress towards this pre-determined end point (Chia, 1978; Pechenik, 1980, 1984). Similar findings have been reported for larval amphibians (Smith-Gill and Berven, 1979) and insects (Nijhout and Williams, 1974), although we do not yet know which, if any, of the physical and hormonal mechanisms responsible for terminating larval life in these organisms apply to the metamorphosis of marine invertebrates (Hadfield, 1978; Highnam, 1981; Pechenik, 1984).

Although the correlation between growth rate and maximum length of larval life was significant for larvae of *C. fornicata,* a great deal of scatter in the data was observed; less than 50% of the variation in the timing of spontaneous metamorphosis was explained (statistically) by variation in estimated individual growth rates (Pechenik, 1984). I suggested that the scatter may reflect a limited correspondence between rates of growth and rates of differentiation, as demonstrated for amphibian development by Smith-Gill and Berven (1979). I could not discuss the relationship between rates of growth and differentiation in *Crepidula* since the larvae were reared in batch cultures and the development of individuals could not be followed. There was also some uncertainty as to whether growth of larval *C. fornicata* was constant throughout the development or whether it declined sometime prior to spontaneous metamorphosis (Pechenik, 1984). Again, the question could not be resolved because larvae were reared in batch cultures, and individual growth could not be followed.

In this paper we report on the development of individually-reared larvae of *C. fornicata.* In particular, we examine the relationships between growth rates, rates of morphological differentiation, and length of planktonic life.

MATERIALS AND METHODS

Adult *Crepidula fornicata* were collected from Woods Hole and Nahant, Massachusetts, and maintained in the laboratory at 20–22°C on a mixed diet of *Dunaliella tertiolecta* and *Isochrysis galbana* or a Tahitian strain of *I. galbana* (T-Iso). All larvae used in this study were released on a single day (Day 0), but not necessarily from a single adult. Metamorphosis, signaling the irrevocable transition from a planktonic to a benthic habitat, was defined by loss of the ciliated velum. Individual larvae were maintained through spontaneous metamorphosis on a uni-algal diet of *I. galbana* or T-Iso at a cell density of approximately 18×10^4 cells per ml in sea water collected from Nahant, Massachusetts, and filtered to 0.45 μm. Larvae were reared individually in glass dishes containing 40–50 ml of algal suspension. Each day, water and food were changed and dishes were thoroughly cleaned with cleanser and acid. Larvae were held under constant light in incubators stable to 0.1°C (Percival Manufacturing).

Rates of growth and morphological differentiation were examined as a function of rearing temperature and diet in this study. Four experiments were conducted over a 14°C range of temperatures (15–29°C). In each experiment, 10–15 larvae were reared at each temperature. The shell length of each larva was measured at 1–2 day intervals at 63× using a dissecting microscope equipped with an ocular micrometer. Growth rates were determined by two different methods (Pechenik, 1984). Average individual growth rates were estimated from the amount of shell growth which occurred between release from the parent and metamorphosis. This was divided by the total length of larval life, providing an estimated average growth rate in μm/day. Average growth rates were also calculated by regressing shell length over time for the first

7–10 days of larval life for each larva. Data were analyzed by One-way Analysis of Variance followed by Fisher's test of Least Significant Difference for comparisons among pairs of means (Ott, 1977). Slopes of regression lines were analyzed according to Kleinbaum and Kupper (1978). Statistical analyses were completed with the aid of the Statistical Package for the Social Sciences (SPSS) (Nie *et al.,* 1975).

The development of two conspicuous morphological features were monitored in these experiments. The shell geometry of *C. fornicata* shifts from a spiral conformation to a more flattened, linear pattern of shell growth during larval life. In particular, a shelf ("brim") develops at the rear of the shell (Pechenik, 1980; Thiriot-Quievreux and Scheltema, 1982). The shell size and date at which this shell brim formed was recorded for each individual larva. The shell length and date at which gill rudiments became apparent were also recorded. These characteristics (brim development and formation of gill rudiments) appear to be the only major morphological alterations which can be monitored non-destructively in larvae of *C. fornicata.*

Most larvae in these experiments ceased growth for one to several days before the occurrence of spontaneous metamorphosis. Feeding rates were determined after growth stopped for three individuals at 20°C and three at 25°C, by determining the rate of disappearance of algal cells from a known volume of algal suspension; appropriate controls were included (Pechenik and Fisher, 1979; Pechenik, 1980). Individual larvae which had stopped growing for two days were placed in a 1.1 ml suspension of *I. galbana* at an initial cell density of 16.1×10^4 cells per ml for 6 h in dim light. Cells per ml were determined using a hemacytometer. Feeding rates were expressed as number of cells eaten per h per larva, for comparison with previous determinations (Pechenik, 1980). Data obtained from the few individuals that metamorphosed during the determination of feeding rate were excluded from analysis. The average shell length (±S.D.) of the larvae (at T_0) used in this experiment was 1032.4 ± 25.2 μm (n = 6).

Biomass determinations were made on 10 individuals following their metamorphosis to establish the relationship between shell length and tissue weight. This relationship is known to be linear for larvae of this species (Pechenik, 1980, 1984). Individuals which had been reared at 24°C were maintained on a diet of *I. galbana* for three to seven days following spontaneous metamorphosis. The juveniles were then preserved in buffered formalin for later determinations of shell length and tissue weight. Dry tissue weights were determined by decalcifying the shells in 2% HCl, then rinsing the tissue free of salts with deionized water and transferring the tissue into pre-weighed aluminum pans (Pechenik, 1980). After 48 h of desiccation over indicating $CaSO_4$ (Drierite), dry tissue weight was determined using a Cahn Model 21 electrobalance in the presence of additional desiccant.

RESULTS

Survivorship and rates of development

Larval survivorship was high. Only one or two individuals died in most treatments. However, at 15°C, mortality was 70% on a diet of T-Iso, compared with 0% on a diet of the non-tropical strain. These results suggest that 15°C is very close to the lowest temperature tolerated by larvae of *Crepidula fornicata;* alternatively, there may be subtle nutritional differences between the two strains of *I. galbana* at different temperatures.

There was generally good agreement between the two methods of estimating rates of shell growth (Table I). Where major discrepancies exist (*e.g.,* 25°C, experiments II and IV), average growth rates as determined from sizes at metamorphosis were

TABLE I

Rates of growth and differentiation before and after spontaneous metamorphosis as a function of food and temperature

Diet	Expt. no.	Rearing temp°C	Growth rate (μm/day) from size at metamorphosis	Growth rate (μm/day) from linear regression	Days to gill	Days to brim	Days to metamorphosis	Growth rate μm/day after metamorphosis
I. galbana	I	18	40.1 ± 9.4 (n = 10)	37.6 (n = 8)	8.5 ± 1.9 (n = 13)	12.1 ± 1.9 (n = 13)	26.5 ± 5.0 (n = 10)	—
				*	*	*	*	
I. galbana	I	24	55.5 ± 9.1 (n = 10)	65.7 (n = 8)	5.5 ± 1.4 (n = 15)	6.6 ± 1.6 (n = 14)	17.6 ± 2.8 (n = 10)	—
I. galbana	II	15	27.9 ± 3.3 (n = 8)	33.3 (n = 5)	9.9 ± 1.3 (n = 11)	16.2 ± 1.3 (n = 12)	28.5 ± 2.4 (n = 10)	—
				*	*	*		
I. galbana	II	20	46.0 ± 13.5 (n = 10)	50.9 (n = 6)	6.4 ± 1.0 (n = 13)	8.2 ± 0.7 (n = 13)	20.5 ± 6.5 (n = 12)	52.5 ± 4.6 (n = 2)
				*	*	*		
I. galbana	II	25	63.5 ± 9.1 (n = 10)	83.2 (n = 5)	4.4 ± 0.9 (n = 13)	6.2 ± 0.4 (n = 13)	12.7 ± 4.8 (n = 11)	116.7 ± 31.8 (n = 9)
I. galbana	II	29	67.2 ± 28.2 (n = 12)	97.5 (n = 6)	3.5 ± 0.5 (n = 13)	5.5 ± 0.7 (n = 13)	12.3 ± 4.1 (n = 12)	89.1 ± 29.2 (n = 7)
T-Iso	III	15	—	15.5 (n = 5)	12.1 ± 1.4 (n = 2)	—	23.0 (n = 1)	—
				*	*			
T-Iso	III	20	40.6 ± 10.4 (n = 6)	52.3 (n = 6)	7.1 ± 1.3 (n = 9)	8.75 ± 0.5 (n = 8)	23.0 ± 5.7 (n = 6)	93.6 (n = 1)
T-Iso	III	25	57.5 ± 18.3 (n = 8)	63.4 (n = 6)	6.6 ± 1.1 (n = 10)	7.9 ± 0.9 (n = 10)	17.9 ± 5.1 (n = 8)	133.1 (n = 1)
T-Iso	IV	20	24.4 ± 4.8 (n = 3)	38.5 (n = 6)	9.2 ± 0.8 (n = 5)	12.0 ± 1.2 (n = 5)	34.7 ± 5.1 (n = 3)	—
				*	*	*		
T-Iso	IV	25	40.4 ± 11.1 (n = 8)	71.4 (n = 6)	5.0 ± 1.6 (n = 11)	7.3 ± 1.9 (n = 11)	19.9 ± 4.0 (n = 9)	128.8 ± 45.9 (n = 4)
T-Iso	IV	29	28.9 ± 14.2 (n = 8)	74.2 (n = 6)	4.3 ± 0.8 (n = 12)	8.3 ± 3.8 (n = 12)	21.1 ± 3.9 (n = 8)	—

* Indicates signficant differences between means ($P < 0.05$, Sheffe's test).

always less than those determined directly from repeated measurements of shell length made on individual larvae (Table I). Note that the discrepancy observed at 25°C in experiment III was relatively small.

Individual growth was reasonably linear over time until larvae reached shell lengths of approximately 900–1100 μm; about 75–100% of the larvae in each treatment then abruptly stopped growing for a number of days prior to spontaneous metamorphosis (Table II). Temperature had no obvious effect on the duration of this hiatus in shell growth. Feeding continued during the period of suspended growth. The average feeding rate (± one S.D.) of six larvae from experiment II (Table I) which had stopped growing for two days was $1.03 \times 10^4 \pm 0.50 \times 10^4$ cells of *I. galbana* eaten per larva per h. This is comparable to feeding rates determined previously for *C. fornicata* larvae of similar size (~1032 μm), and is less than the average feeding rate for competent larvae of ~800–900 μm (Pechenik, 1980).

Following spontaneous metamorphosis, shell growth occurred for individuals in all treatments, except at 29°C in experiment IV. In this treatment, all post-metamorphic individuals died. A temperature of 29°C is near the lethal limit for larvae of *C. fornicata* (Lucas and Costlow, 1979). In all other treatments, post-metamorphic shell growth was a linear function of time, and growth resumed at rates equal to or exceeding rates of shell growth recorded for the larvae (Table I). The relationship between shell length (Y) and tissue biomass (X) for post-metamorphic individuals reared at 24°C was found to be linear: $Y = 0.043X - 36.28$, $r^2 = 0.76$. The implication is that for at least one week following spontaneous metamorphosis, constant growth resumes in terms of both shell length and biomass.

Increased temperature generally accelerated the rate of shell growth. The slopes of the lines relating larval shell length to days in culture were significantly different ($\alpha = 0.05$) from each other at both temperatures in experiment I and for the lowest three temperatures of experiment II; larvae were fed *I. galbana* in both experiments (Table I). In experiment III, rates of shell growth for larvae fed T-Iso were significantly different at 15 and 20°C, but were not significantly different at 20 and 25°C. In experiment IV, in which the diet was also T-Iso, shell growth rates were significantly different from each other at 20 and 25°C, but not at 25 and 29°C (Table I).

All conditions which altered rates of shell growth also altered time to formation of gill rudiments and shell brims, and maximum length of larval life ($P < 0.05$) (Table I). Conditions which did not alter growth rates did not alter length of planktonic life or time to brim and gill formation. For example, in experiment II (Table I), growth rates were essentially the same at 25 and 29°C. Length of larval life, time to appearance of shell brims, time to appearance of gill rudiments were also comparable for larvae reared at these two temperatures. In the same experiment, however, growth rates recorded at 15 and 20°C were significantly different from those recorded at the higher temperatures, and were significantly different from each other as well. These differences in growth rates were correlated with differences in time to spontaneous metamorphosis, and in time to formation of gill rudiments and shell brims (Fig. 1; Table I).

Effect of temperature on the relative rates of growth and differentiation

Over the range 18–25°C, temperature did not affect ($\alpha = 0.05$) the sizes at which gill rudiments or shell brims first became visible, or at which spontaneous metamorphosis occurred (Table III). However, mean shell length at first appearance of gill filaments was significantly reduced at 15°C in experiment II, and both shell length at first appearance of gill filaments and size at metamorphosis were significantly reduced at 29°C in experiment IV. Rates of growth and differentiation (at least with

TABLE II

Cessation of growth prior to spontaneous metamorphosis

Diet	Expt. no.	Temperature (°C) 15°	18°	20°	24°	25°	29°
I. galbana	I	—	1.9 ± 1.9 (100%) n = 10	—	4.6 ± 3.0 (70%) n = 10		—
I. galbana	II	4.4 ± 2.0 (80%) n = 8	—	4.0 ± 3.4 (83%) n = 10	—	1.4 ± 1.4 (73%) n = 10	2.6 ± 2.5 (75%) n = 12
T.-Iso	III	3.0 (100%) n = 1	—	3.5 ± 2.4 (100%) n = 6	—	3.8 ± 2.8 (87%) n = 8	—
T.-Iso	IV	—	—	6.0 ± 1.7 (100%) n = 3	—	7.1 ± 2.9 (100%) n = 9	8.9 ± 3.9 (100%) n = 8

Data are average number of days (±S.D.) that growth stopped.
Percentage of larvae which ceased growth for ≥1 day is given in parentheses for each treatment.

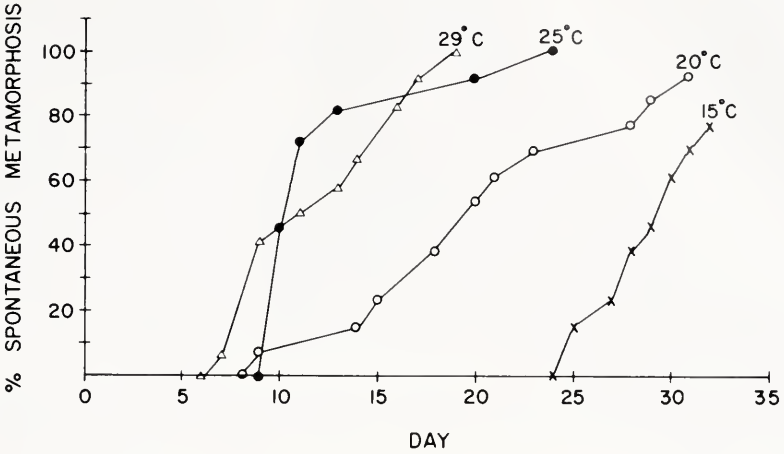

FIGURE 1. The influence of temperature on the number of days elapsed between larval release and spontaneous metamorphosis of *C. fornicata.*

respect to gill differentiation) thus appear to be differentially sensitive to major changes in temperature.

Relationship between growth rate, differentiation rate, and length of planktonic life

Average rates of morphological differentiation were expressed as (days to first appearance of gill filaments)$^{-1}$ and as (days to first appearance of a shell brim)$^{-1}$.

TABLE III

Influence of temperature on size at which morphological features develop and size of spontaneous metamorphosis

Diet	Expt.	Rearing temp. °C	Mean size at gill formation	Mean size at brim formation	Mean size at spontaneous metamorphosis
I. galbana	I	18	767.7 ± 48.0 (n = 13)	900.6 ± 70.3 (n = 13)	1475.4 ± 118.6 (n = 10)
I. galbana	I	24	784.6 ± 58.9 (n = 15)	881.6 ± 89.1 (n = 14)	1404.5 ± 57.1 (n = 10)
I. galbana	II	15	637.5 ± 30.5 (n = 11)	843.7 ± 71.2 (n = 12)	1167.9 ± 94.5 (n = 8)
			*		
		20	704.2 ± 23.3 (n = 13)	863.6 ± 62.7 (n = 13)	1237.0 ± 166.8 (n = 10)
		25	700.3 ± 33.4 (n = 13)	905.5 ± 68.2 (n = 13)	1095.2 ± 92.8 (n = 10)
		29	717.0 ± 40.5 (n = 13)	907.0 ± 73.5 (n = 13)	1111.2 ± 109.7 (n = 12)
T-Iso	III	15	747.1 ± 32.9 (n = 2)	—	977.4 (n = 1)
		20	779.5 ± 57.6 (n = 9)	897.0 ± 37.4 (n = 8)	1302.7 ± 100.2 (n = 6)
		25	817.3 ± 76.8 (n = 10)	961.4 ± 59.9 (n = 10)	1364.2 ± 71.5 (n = 8)
T-Iso	IV	20	812.4 ± 36.9 (n = 5)	916.8 ± 53.7 (n = 5)	1241.2 ± 46.3 (n = 3)
		25	793.4 ± 58.7 (n = 11)	877.8 ± 76.7 (n = 11)	1203.8 ± 249.1 (n = 8)
			*		*
		29	712.2 ± 62.8 (n = 12)	853.9 ± 79.8 (n = 12)	986.1 ± 172.0 (n = 8)

Data are means (μm) ± one S.D.

* Indicates significant differences between means ($P < 0.05$).

Pooling the 120 data points for all temperatures indicates that both measures of differentiation rate are positively correlated with growth rate (Figs. 2a, b). Approximately 75% of the variation in growth rate was explained (statistically) by variation in differentiation rate in both cases.

Pooling the data for the 102 larvae which survived to spontaneous metamorphosis reveals an inverse correlation between the day on which spontaneous metamorphosis occurred (Y) and the rate of growth (X) as determined by periodic measurements of shell length (Fig. 3). The relationship is approximately linear ($Y = 34.83 - 0.23X$, $r = -0.72$) and the slope of the relationship is significantly different from zero ($P < 0.05$). Approximately 50% of the variation in the timing of metamorphosis was explained by variation in shell growth rate. Treating day of spontaneous metamorphosis as a function ln growth rate does not improve the strength of the relationship: $Y = 71.58 - 12.55X$, $r^2 = 0.47$.

The day of spontaneous metamorphosis is inversely related to the rate of morphological differentiation (Figs. 4a, b). If the relationship is treated as linear, ap-

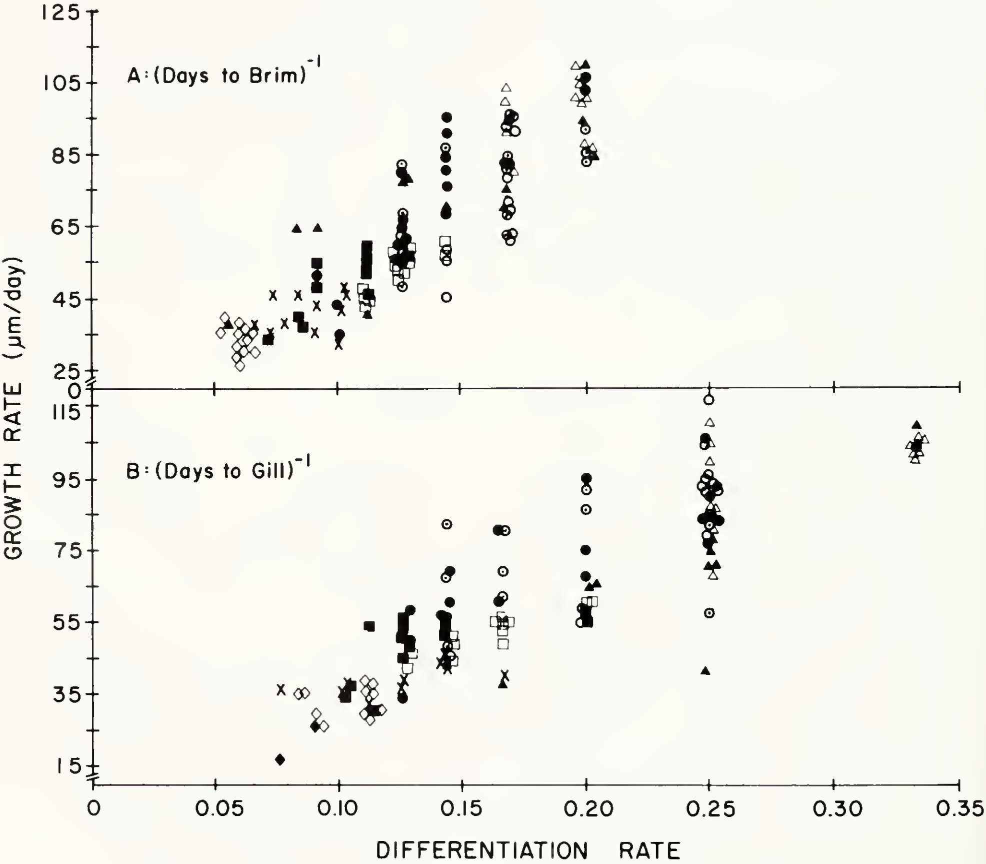

FIGURE 2. Relationship between rates of growth (μm/day) and rates of morphological differentiation (days^{-1}) for larvae of *C. fornicata*. Differentiation rates were measured as (days required to develop a brimmed shell)$^{-1}$ (A) and as (days required to develop conspicuous gill rudiments)$^{-1}$ (B). Legend: *Iso* diet—◇ = 15°C, × = 18°C, □ = 20°C, ⊙ = 24°C, ○ = 25°C, △ = 29°C. *T-Iso* diet—◆ = 15°C, ■ = 20°C, ● = 25°C, ▲ = 29°C.

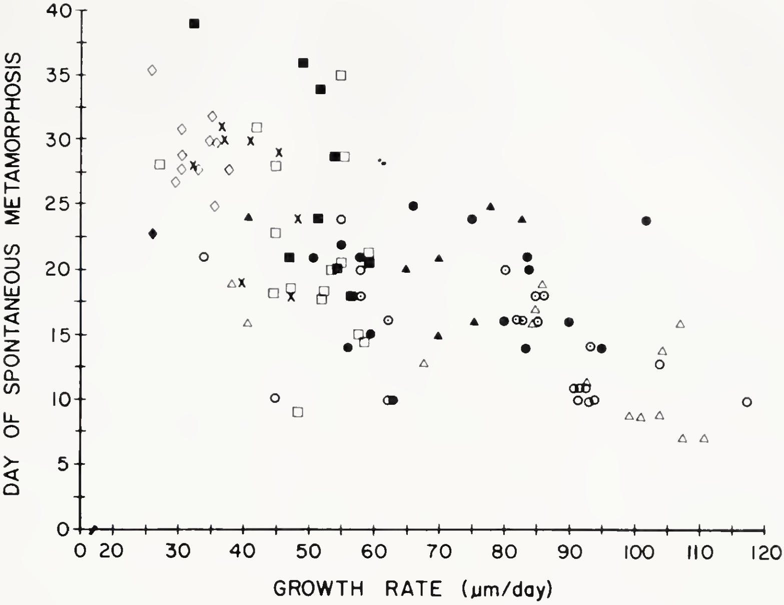

FIGURE 3. Relationship between growth rate and duration of planktonic life for larvae of *C. fornicata*. Symbols are defined in Figure 2.

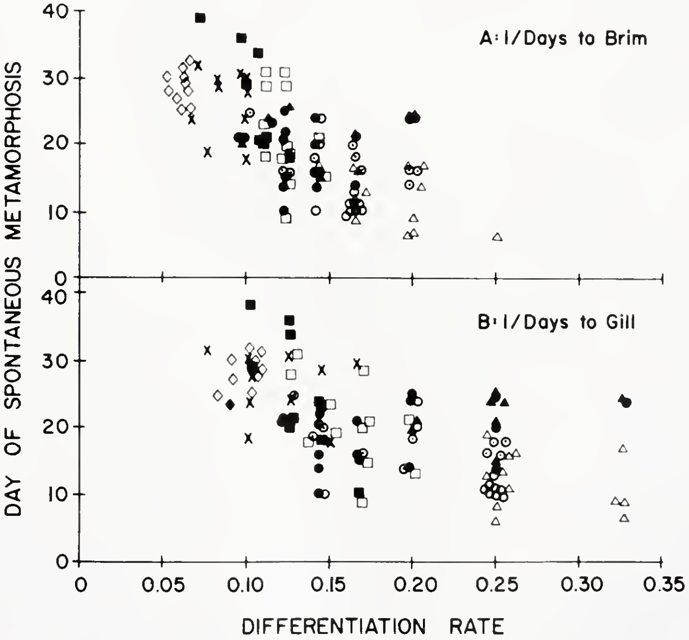

FIGURE 4. Relationship between rates (day^{-1}) of morphological differentiation and duration of larval life in *C. fornicata*. Symbols are as defined in Figure 2.

proximately 46% and 37% of the variation in when metamorphosis occurs is explained by variation in the timing of brim formation and gill formation, respectively. Considering the relationship to be logarithmic, approximately 48% and 42% of the variation in the timing of metamorphosis is explained by variation in differentiation rate.

DISCUSSION

Larvae of most marine invertebrates studied to date have the capacity to prolong the planktonic stage beyond the point at which metamorphosis first becomes possible (Crisp, 1974; Scheltema, 1974). Although most studies on the induction and delay of metamorphosis have been conducted in the laboratory, there is evidence that the larvae of several mollusc species also delay metamorphosis in the field (Bayne, 1965; Scheltema, 1971; Pechenik, 1978). The ecological significance of this ability has often been pointed out (*e.g.*, Scheltema, 1961; Bayne, 1965; Crisp, 1974; Doyle, 1975; Obrebski, 1979; Jackson and Strathmann, 1981). However, few studies have been concerned specifically with defining ability to delay metamorphosis for any particular species, or determining the factors which affect delay capability.

Bayne (1965), working with larvae of the blue mussel, *Mytilus edulis,* was the first to study the influence of environmental factors on delay potential, and was the first to consider a relationship between rate of growth and duration of the delay period. He found that both salinity and temperature had a pronounced effect on the length of time that metamorphosis could be delayed by mussel larvae. However, no correlation between rate of growth to the pediveliger stage and length of time that metamorphosis of mussel larvae could be delayed was detected (Bayne, 1965). This is in sharp contrast to results reported here and elsewhere (Pechenik, 1984; Lima and Pechenik, in review) for larvae of *C. fornicata* and *C. plana,* in which slower growth is significantly correlated with a greater capacity for delayed metamorphosis. Absence of such a relationship in the larvae of *M. edulis* is most likely explained by the fact that these larvae exhibit degeneration of the velum and ciliary feeding tracts as metamorphosis is delayed; larvae eventually become unable to feed at all (Bayne, 1965). Delaying larvae of *M. edulis* are thus forced to obtain all nutrients from internal energy stores, and the length of time that metamorphosis can be delayed is limited by the size of these energy stores and the rate at which these reserves are depleted. Environmental factors such as increased temperature and altered salinity presumably increase the rate at which energy stores of delaying mussel larvae are depleted and foreshorten the delay period accordingly. At 10–11°C, for example, a temperature range at which metabolic rates are likely to be low, mussel larvae could postpone metamorphosis for 43–46 days. The delay period was reduced to only two days at 21–22°C, conditions apt to produce much higher metabolic rates (Vernberg, 1972).

In contrast to larvae of *M. edulis,* larvae of *C. fornicata* (in batch culture) continue to feed, and show no sign of morphological degeneration or energy imbalance after becoming competent to metamorphose in batch culture (Pechenik, 1980). The present paper indicates that even when growth of individuals ceases prior to spontaneous metamorphosis, feeding rates remain high. However, more detailed measurements of feeding rates, respiration rates, and assimilation efficiencies will have to be made for larvae which have stopped growing, to determine whether the cessation of growth in larvae of *C. fornicata* has a nutritive/energetic basis. The abruptness with which growth ceased suggests that dietary insufficiency was not the cause. Growth resumed following spontaneous metamorphosis of all individuals under most temperature conditions, again arguing against a pathological basis for cessation of growth by *C. fornicata* larvae. That growth ceases prior to spontaneous metamorphosis was anticipated from results of experiments with batch cultures of larvae (Pechenik, 1984), but the abruptness of the transition was not.

Some opisthobranch gastropod larvae also cease growth during development, generally prior to the onset of competence (Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977; Kempf, 1981). This state of no-growth can then be maintained for many weeks, or even months, and is presumed to play a role in prolonging larval life in gastropods (Scheltema, 1966; Kempf, 1981). It should be pointed out, however, that growth of *C. fornicata* did not always stop for all individuals within a treatment (Table II), and that even when growth did cease, spontaneous metamorphosis generally occurred within a few days. The significance of the cessation of larval shell growth in *C. fornicata* thus remains unclear. Present indications for larvae reared in batch culture are that the congener *C. plana* does not cease growth prior to spontaneous metamorphosis. That is, for larvae of *C. plana*, growth rates estimated from size at metamorphosis were comparable to growth rates determined by direct measurements made earlier in development (Lima and Pechenik, in review).

The present study confirms the inverse correlation between growth rate and duration of planktonic life reported previously for larvae of *C. fornicata* reared in batch culture, and supports the hypothesis that length of larval life in this species is limited by the rate of development towards a pre-determined end point (Pechenik, 1984). The same must also be true of the larvae of *Mytilus edulis;* the immediate cause of the end to larval life in that species may well be nutritional limitation (Bayne, 1965), but the loss of feeding ability itself would seem to be the result of a developmentally programmed degeneration of larval structure.

The ability to predict when spontaneous metamorphosis will occur in *C. fornicata* has not been improved by the results of this study. Although growth rates are seen to be well-correlated with rate of progress towards gill development and shell brimming, neither measure of differentiation was better correlated with length of larval life than was growth rate. This is in contrast to results reported for amphibian larvae by Smith-Gill and Berven (1979). As with *Crepidula* larvae, growth rate was found to be a statistically significant, but mediocre predictor of the onset of amphibian metamorphosis. Prediction capabilities for larval amphibians improved dramatically when rate of morphological development was monitored instead of growth rate. The tight correlation between rate of morphological development and date of metamorphosis obtained in their study is explained by the fact that the morphological changes measured are controlled by the same hormonal mechanism responsible for metamorphosis (Smith-Gill and Berven, 1979). Most likely, the factors responsible for brim formation or gill proliferation in delaying larvae of *C. fornicata* have little to do with progress towards the point at which the velum is lost. Additional interpretation must await clarification of the internal mechanisms involved in terminating larval life in marine invertebrates.

Kempf's (1981) study also focused on the duration of delayed metamorphosis for a gastropod species with feeding larvae. In his experiments with larvae of the opisthobranch *Aplysia juliana*, a few individuals survived as long as 316 days after release from the egg mass, and showed no sign of morphological degeneration or loss of ability to metamorphose in response to inducer. However, most of the larvae died during the experiment; mortality was approximately 50% during the first eight weeks of laboratory culture. As the author suggests, mortality may have resulted from inadequate culture conditions, perhaps a dietary insufficiency. Relative incidence of mortality and spontaneous metamorphosis in molluscan larvae may also be affected by temperature regime (Pechenik, 1984a). However, the possibility that the mortality of delaying opisthobranch larvae is somehow developmentally programmed, *i.e.*, that there is a larval endpoint, cannot be ruled out. Since mollusc larvae develop at a variety of different rates within a single laboratory culture (*e.g.,* Bayne, 1965; Hickman and Gruffydd, 1971; Lucas and Costlow, 1979; Pechenik, 1984), the logarithmic mortality curve reported by Kempf (1981) may in part reflect such differences in rates of development towards the hypothesized larval end point. Possibly the few larvae which survived the 316 days are those which developed the most slowly throughout the study; because larvae were reared in batch culture, individual rates of development could not be followed. Even had the larvae been reared individually, rates of differentiation are likely to be better predictors of delay periods than rates of growth (Pechenik, 1984), and these are not easily measured. Moreover, as in the present study, actual delay periods cannot be known with certainty because the time at which an individual larva first becomes competent to metamorphose can be determined only by inducing metamorphosis (Pechenik, 1984). Our ability to further explore the proximal factors controlling delay of metamorphosis by gastropod larvae is thus hindered by the difficulty of ascertaining individual competence and monitoring rates of individual differentiation non-destructively. The ideal species for future study will be one demonstrating a pronounced morphological or behavioral correlate of competence, and a series of discrete morphological or behavioral changes which are under the influence of the same internal factors responsible for the termination of larval life.

ACKNOWLEDGMENT

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GENE FLOW IN MAINLAND AND INSULAR POPULATIONS OF *CRASSOSTREA* (MOLLUSCA)

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ABSTRACT

Insular and mainland populations of *Crassostrea* species were compared with respect to relative gene flow, levels of genetic variation, and population differentiation. The relative level of gene flow was reduced among Caribbean Island subpopulations as compared to mainland subpopulations along the Atlantic seaboard and Gulf of Mexico. However, this reduction in gene flow among island subpopulations has resulted in very little population differentiation between islands or between islands and mainland demes. This suggests that the existing level of insular gene flow may adequately override genetic drift, founder effect, and weak selection. The reduction in gene flow was, however, accompanied by a substantial drop in the average heterozygosity among island demes when compared to mainland populations.

INTRODUCTION

There are two opposing views concerning the significance of gene flow among natural populations. One proposes that gene flow is a conservative force which prevents population differentiation (Mayr, 1963; Dobzhansky, 1970; Stanley, 1979). The other holds that gene flow is ineffective and that populations which freely exchange genes may indeed differentiate as long as there exists different selection regimes within the distribution of a species (Ehrlich and Raven, 1969). Perhaps both views are partially correct. Recently an experimental method of studying gene flow among natural populations was developed. Slatkin (1981) has derived a method of estimating relative gene flow among natural populations by using the allele frequency data from protein gel-electrophoretic studies. He found that gene flow differs greatly among a wide range of species. Among populations of *Drosophila* species, the blue mussel, milkfish, and an annual plant, he found high levels of gene flow which he considers make such species nearly panmictic. For these species gene flow appears to be a conservative force, and a species may be considered as a single evolutionary unit as predicated by Mayr (1963), Dobzhansky (1970), and Stanley (1979). In contrast, Slatkin found salamanders to have relatively low levels of gene flow. Consequently, Slatkin's data gathered on the salamander species supported the views of Ehrlich and Raven (1969). Since the evidence supports two contrasting views on the importance of gene flow with reference to population differentiation, the two views were examined by comparing the effects of gene flow among contiguous shoreline populations with those of island populations. An outbreeding marine species with good larval dispersing capabilities was selected for study.

The study compares levels of gene flow among insular and contiguous mainland populations of marine pelecypod species of the genus *Crassostrea* which, like the blue mussel, *Mytilus edulis,* have long planktonic larval stages. There is reason to

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believe that a reduction in inter-island gene flow should occur. The constraints upon pelagic larval dispersion along continental coasts are few. The direction and velocity of water currents and the time of reproduction and length of pelagic life are the only limitations placed upon larval dispersion among mainland populations (Scheltema, 1977). In addition to these constraints, insular pelagic larvae will experience other limitations. Most pelagic larvae aggregate in the plankton and are transported in swarms. Consequently, if an island is small, larval swarms may pass by it without making contact (Thorson, 1961) thereby leading to sporadic inter-island population disjunction in gene flow. If differences in the level of gene flow can be found, what effect might this have on the levels of genetic variation and population differentiation within the species distribution?

The American oyster, *C. virginica,* is distributed along the eastern seaboard of North America and the Gulf of Mexico. South of the Yucatan peninsula and in the Caribbean Islands, the mangrove oyster, *C. rhizophorae,* replaces *C. virginica* (Stenzel, 1971). Both species have planktonic larval stages which last from two to three weeks providing ample opportunity for zygotic dispersion (Galtsoff, 1964). These species are considered to be very closely related and some workers consider them to be different varieties of the same species, *C. virginica* (Mattox, 1949; Galtsoff, 1964; Menzel, 1968; 1971). The two species have been hybridized in the laboratory with the F_1 generation showing an integration of both parental types (Menzel, 1973). Perhaps the major physiological difference between the two species is that *C. virginica* is considered a euhaline species while *C. rhizophorae* is a hyperhaline species (Gunter, 1951). The allozyme data is now available for both species so that Slatkin's (1981) method of estimating gene flow can be used to examine the relative level of gene flow among the insular and mainland populations.

MATERIALS AND METHODS

The mainland populations of *Crassostrea* species were from the Chesapeake Bay, the Atlantic seaboard, and the Gulf of Mexico (Buroker, 1983, 1984). Insular populations of *C. rhizophorae* consist of groups from the Dominican Republic, Guadeloupe Island, Puerto Rico (Hedgecock and Okazaki, 1984), and the Virgin Islands (Buroker *et al.,* 1979). The population groups were (1) Chesapeake Bay (10 collecting localities or subpopulations), (2) the Atlantic coast and Gulf of Mexico (18 subpopulations), and (3) Caribbean Islands (4 different islands). The collecting locales were considered subdivisions of a single population (area) to estimate the relative levels of gene flow within each of the three areas. Thirty homologous structural loci as detected by protein electrophoresis were compared among these populations (Appendix).

RESULTS

A list of the monomorphic and polymorphic structural loci for *Crassostrea* species from each of the three areas is provided (Appendix). The heterozygosities for the polymorphic loci and the overall average heterozygosities of the *Crassostrea* species from each area are also given (Appendix). Slatkin's (1981) procedure for estimating gene flow among natural populations was applied. Slatkin's conditional average frequency, $\bar{p}_{(i)}$, was computed for the allozyme data among the subpopulations from each of the three areas. For each area, conditional average frequencies were plotted against i/d, where i ranges from one to d, and d is the number of subpopulations (Fig. 1, Table I). Although this procedure is a qualitative rather than a quantitative technique, it can be used to determine relative differences in levels of gene flow

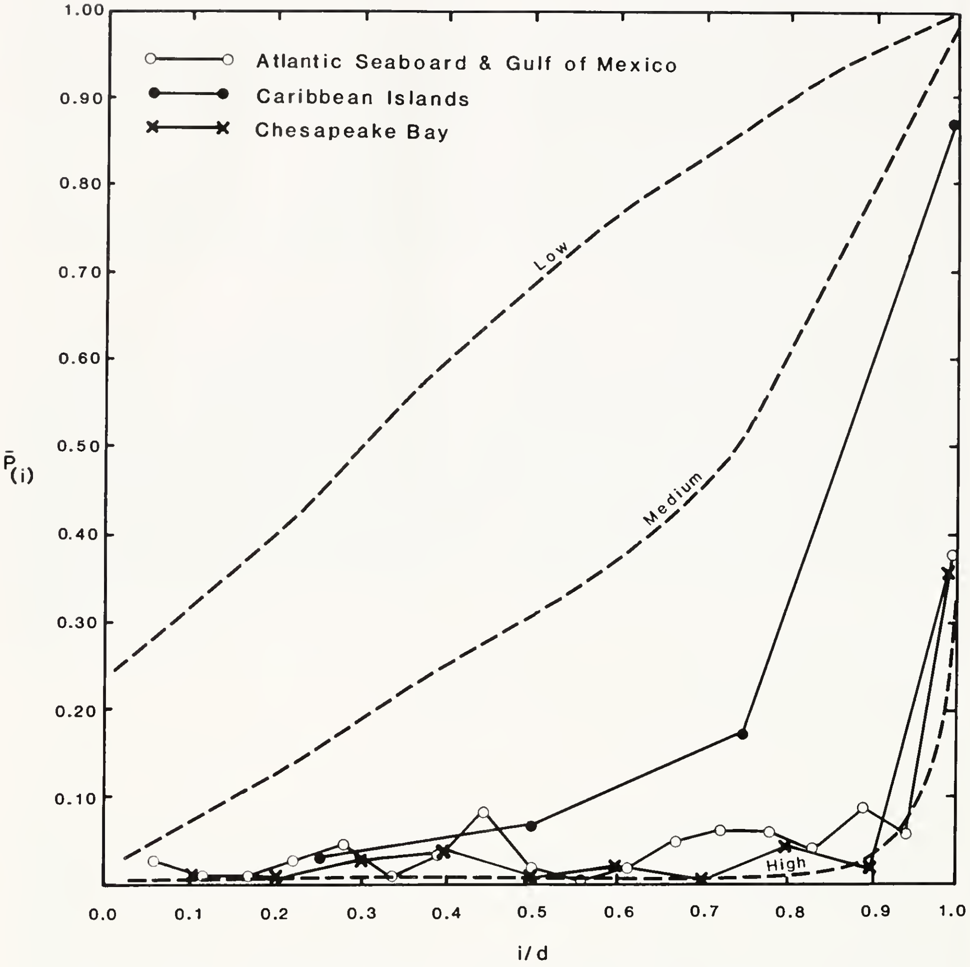

FIGURE 1. Plot of conditional average frequencies for different geographical regions of *Crassostrea* species. In each case $\bar{p}_{(i)}$ is plotted against i/d, where d is the number of sampling localities, to permit a comparison of mainland and insular populations. The dashed line depicts levels of gene flow among hypothetical populations.

between the three areas. Nei's (1975) coefficient of gene differentiation (G_{st}) for two $\bar{p}_{(i)}$ values was computed from each area (Table II). The coefficient can vary from zero (no gene differentiation) to one (complete gene differentiation) and provides information on the relative magnitude of gene differentiation among subdivisions within a population. It is used to compare the effects of gene flow between the Chesapeake Bay, the Atlantic Coast/Gulf of Mexico, and the Caribbean Island populations of *Crassostrea* species. The coefficient of gene differentiation can be related to Slatkin's (1981) procedure of estimating gene flow when the conditional average frequency of a given set of i/d intervals is used to determine the G_{st} value. In this instance, areas which have similar G_{st} values would presumably have similar levels of gene flow among their subpopulations while those with dissimilar G_{st} values would have a different level of gene flow among their subpopulations.

To compute the conditional average frequencies, $\bar{p}_{(i)}$, from allozyme data of three hypothetical species with low, medium, and high levels of gene flow, the plots of $\bar{p}_{(i)}$ with the appropriate i/d interval would appear as the broken line curves of Figure 1 (Slakin, 1981). In Figure 1, the conditional average frequencies are plotted against i/d from Table I as computed from the allozyme data of *Crassostrea* species from the Caribbean Islands (Buroker *et al.*, 1979; Hedgecock and Okazaki, 1984), the Chesapeake Bay (Buroker, 1984), and the Atlantic Coast and Gulf of Mexico (Buroker, 1983). Figure 1 shows little difference in the level of gene flow among collecting localities from the Chesapeake Bay as compared to those along the Atlantic seaboard

TABLE I

Geographical areas where Crassostrea *species were studied and background information for the data plotted in Figure 1*

	Caribbean Islands		Chesapeake Bay		Atlantic Coast & Gulf of Mexico	
i	i/d	$\bar{p}_{(i)}$	i/d	$\bar{p}_{(i)}$	i/d	$\bar{p}_{(i)}$
1	0.25	0.029 ± .020	0.10	0.008 ± .003	0.056	0.029 ± .060
2	0.50	0.065 ± .044	0.20	0.006 ± .001	0.111	0.010 ± .004
3	0.74	0.171 ± .125	0.30	0.028 ± .032	0.167	0.010 ± .004
4	1.00	0.870 ± .134	0.40	0.037 ± .043	0.222	0.043 ± .001
5			0.50	0.009 ± .001	0.278	0.045 ± .063
6			0.60	0.021 ± .027	0.333	0.010 ± .002
7			0.70	0.000	0.389	0.033 ± .034
8			0.80	0.043 ± .000	0.444	0.105 ± .163
9			0.90	0.019 ± .014	0.500	0.020 ± .003
10			1.00	0.362 ± .313	0.556	0.000
11					0.611	0.020 ± .016
12					0.667	0.049 ± .030
13					0.722	0.062 ± .074
14					0.778	0.061 ± .079
15					0.833	0.042 ± .028
16					0.889	0.092 ± .000
17					0.944	0.057 ± .000
18					1.000	0.381 ± .352
d		4		10		18
Polymorphic loci used*		6		16		21
Alleles		23		79		109
Range of sample size		4–54		36–93		27–138
Apparent level of gene flow		Med/High		High		High
Reference		Buroker *et al.*, 1979 Hedgecock and Okazaki, 1984		Buroker, 1984		Buroker, 1983

The data presented are the conditional average frequencies ($\bar{p}_{(i)}$ ± S.D.) for each interval (i/d), the number of sampling localities (d), the number of polymorphic loci studied, the number of alleles used, the range of sample size from each area and the apparent level of gene flow.

* Although other polymorphic loci are listed in the Appendix, not all were represented among every sampling locality; and therefore, could not be used in constructing Figure 1.

and Gulf of Mexico. They both have high levels of gene flow among subpopulations and are in agreement with Slatkin (1981) for *M. edulis* along the Atlantic seaboard. Among insular Caribbean subpopulations the plot of $\bar{p}_{(i)}$ against i/d falls between a medium and high level of gene flow (Fig. 1). This suggests that mainland populations of *Crassostrea* species have higher levels of gene flow than insular populations. To substantiate this result among the three study areas, the coefficient of gene differentiation (G_{st}) was computed for two corresponding conditional average frequencies of each area (Table II). If gene flow were less among island demes than mainland demes, a larger coefficient of gene differentiation among island demes than mainland communities would be expected. This was substantiated by the G_{st} results (Table II). Subpopulations in the Chesapeake Bay, the Atlantic coast, and Gulf of Mexico have approximately the same amount of gene differentiation (*i.e.*, very little). The lack of gene differentiation between these subpopulations results from similarly high levels of gene flow which overrides the other evolutionary forces of drift and weak selection. Table II also indicates that insular demes have greater gene differentiation than the other two areas. This suggests that less gene flow occurs between island demes than among contiguous mainland subpopulations.

The effect these factors have on levels of genetic variation within conspecific subpopulations proves interesting. Using average heterozygosity to estimate genetic variation, a great reduction in this estimate accompanies the reduction in gene flow. Individual heterozygosities, averaged over all loci, were approximately 22 and 24% for subpopulations in the Chesapeake Bay and along the Atlantic Coast/Gulf of Mexico, respectively. The same estimate averaged 12% within the Caribbean Island subpopulations (Appendix). This suggests that among these *Crassostrea* species a reduction in gene flow in conjunction with subpopulation inbreeding reduces the genetic variation necessary for evolutionary change. The lower average heterozygosities of insular populations of *C. rhizophorae* may not be species specific, since heterozygosities within mainland populations of *C. rhizophorae* (Hedgecock and Okazaki, 1984) and *C. virginica* (Buroker, 1983, 1984) are similar.

DISCUSSION

Low levels of genetic variation within Caribbean Island subpopulations of *C. rhizophorae,* as compared to mainland subpopulations of *Crassostrea,* may result from genotypic adaptations to oceanic islands. From this we might expect genetic differentiation between mainland and insular oysters. According to Hedgecock and Okazaki (1984) the amount of genetic differentiation is not great. They report the genetic similarity (Nei, 1978) among *C. rhizophorae* populations to be 0.97 between Santo Domingo in the Greater Antilles and Belize, Mexico and 0.95 between Santo

TABLE II

A summary of genetic parameters studied among three geographical areas of Crassostrea *species*

Area	d	i/d	$\bar{p}_{(i)}$	G_{st}
Chesapeake Bay	10	0.800	0.043	0.001
		1.000	0.362	0.002
Atlantic Coast & Gulf of Mexico	18	0.778	0.061	0.008
		1.000	0.381	0.004
Caribbean Islands	4	0.750	0.171	0.013
		1.000	0.870	0.041

The data presented are the number of collecting localities or subpopulations (d), the i/d intervals, the conditional average frequencies [$\bar{p}_{(i)}$] and the corresponding coefficient of gene differentiation (G_{st}).

Domingo and Trinidad. Even though gene flow is reduced among Caribbean Island subpopulations, it is still adequate in conjunction with the large insular demes to over-ride genetic drift, founder effects, and perhaps weak selection. A second explanation for low levels of genetic variation within the Caribbean Island subpopulations could result if the reduction in gene flow among subpopulations produces an imbalance between the occurrence of new alleles in the deme and the degree of inbreeding. If the insular deme size remains constant, the level of gene flow might effect the balance between the occurrence of new alleles and the degree of inbreeding within a subpopulation. For example, a high degree of gene flow in conjunction with a constant mutation rate would override the effects of inbreeding within a deme and the insular subpopulation would experience an influx of new alleles producing a relatively high level of genetic variation. In contrast, assuming the mutation rate remains the same, the situation could be reversed if gene flow were reduced. That is, inbreeding would eliminate alleles and erode away genetic variation within a deme. A third explanation for the low level of genetic variation among the Caribbean Island demes could result from the small sample size used in computing the average heterozygosities (Buroker *et al.,* 1979; Hedgecock and Okazaki, 1984). Smaller sample sizes are certainly biased against rare alleles. However, two independent studies (Buroker *et al.,* 1979; Hedgecock and Okazaki, 1984) provide standard errors with their estimates. These studies largely coincide and agree that average heterozygosity is significantly lower within insular demes when compared to mainland subpopulations. The final explanation concerns the insular deme size. With the mutation rate constant, we can assume that the frequency of neutral mutations maintained within a deme is directly related to its size (Crow and Kimura, 1970), and small demes should have lower levels of genetic variation then larger ones. This explanation can not be discounted since an accurate determination of the number of breeding individuals within a subpopulation is not available.

The smallest, most distant islands from the coastal mainland have the lowest levels of genetic variation among natural *C. rhizophorae* populations. For example, Puerto Rico, the Virgin Islands, and Guadeloupe Island have average heterozygosities of 9, 8, and 11%, respectively (Buroker *et al.,* 1979; Hedgecock and Okazaki, 1984). In contrast, the large Caribbean Island of the Dominican Republic, although very distant from coastal mainland populations, has an average heterozygosity of 15% for the endemic Santo Domingo oyster population. These data coincide with the *gene flow-variation* hypothesis described by Soulé (1971) in his study of the side-blotched lizard, *Uta stansburiana,* from the Gulf of California. Soulé assumes that populations throughout a species range are exposed to different selection intensities. Gene flow between these populations causes an exchange of endemic mutations which results in elevated genetic variation throughout the species range. The theory predicts that species differing in levels of gene flow many also differ in levels of genetic variation. For example, a species displaying a high level of gene flow as defined by Slatkin (1981) might maintain a larger number of rare alleles throughout its distribution than a species with a lower level of gene flow among populations. This is precisely what we see when we compare mainland populations of *Crassostrea* species (Buroker, 1983, 1984) with insular populations (Buroker *et al.,* 1979; Hedgecock and Okazaki, 1984). The work of Buroker *et al.,* (1979) and Hedgecock and Okazaki (1984) indicates that each island population has many rare alleles often endemic to that locality. Since these alleles are present in low frequency and levels of gene flow are reduced among insular as compared to mainland demes, these endemic rare alleles are not maintained throughout the Caribbean Island populations as they are among mainland populations of *Crassostrea* species. Consequently, a reduction in genetic variation within insular demes is experienced.

Soulé (1971) points out that natural populations on large islands often cannot be differentiated from mainland populations in their levels of variation. As Soulé shows, there is some asymptotic level of variation that is realized with increasing land area. Among the Caribbean Islands studied by Hedgecock and Okazaki (1984), the Dominican Republic (Santo Domingo) has by far the greatest land area (30,785 sq. km) followed by Puerto Rico (5528 sq. km), Guadeloupe Island (2044 sq. km), and the Virgin Islands (322 sq. km). Consequently, the Santo Domingo oyster subpopulation from the Dominican Republic, with its 15% average heterozygosity, is closer than any other Caribbean Island subpopulation studied to the apparent asymptotic level of 24% heterozygosity realized by mainland *Crassostrea* subpopulations.

In summary, I have presented evidence that relative gene flow is different between mainland and insular populations of *Crassostrea* species. In conjunction it has been observed that the reduction in gene flow accompanies lower levels of genetic variation within insular demes. Although gene flow was found to differ between insular and mainland *Crassostrea* populations, very little subpopulation differentiation (genetic divergence) was found with the decrease in gene flow. These results suggest that although gene exchange between demes is important in maintaining similarity in population structure they also indicate that the reduction in gene flow was not enough to cause genetic divergence among insular demes.

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APPENDIX

Protein loci used for genetic analysis

	Polymorphic loci and their heterozygosity					
	Chesapeake Bay		Atlantic Coast Gulf of Mexico		Caribbean Islands	
Protein locus	d	H	d	H	d	H
Aminopeptidase-1	10	0.765 ± .010	18	0.727 ± .008	1	0.333 ± .045
Acid phosphatase-3	7	0.697 ± .014	18	0.616 ± .009	1	0.479 ± .051
Adenylate kinase-1	10	0.614 ± .012	18	0.432 ± .009	3	0.259 ± .030
Aspartate aminotransferase-1	10	0.000	18	0.028 ± .003	4	0.000
Aspartate aminotransferase-2	10	0.388 ± .012	15	0.465 ± .011	4	0.088 ± .017
Aldolase	10	0.000	18	0.012 ± .002	1	0.000
Esterase-1	10	0.809 ± .009	18	0.627 ± .009	1	0.000
Glyceraldehyde 3-phosphate dehydrogenase	10	0.000	18	0.007 ± .001	4	0.000
Isocitrate dehydrogenase-1	10	0.045 ± .005	18	0.053 ± .004	1	0.056 ± .022
Isocitrate dehydrogenase-2	10	0.018 ± .003	18	0.038 ± .003	1	0.000
Lecine aminopeptidase-1	10	0.574 ± .012	18	0.608 ± .009	4	0.449 ± .031
Lecine aminopeptidase-2	10	0.357 ± .011	18	0.426 ± .009	1	0.286 ± .046
Malate dehydrogenase-1	10	0.011 ± .002	18	0.008 ± .002	4	0.017 ± .008
Malate dehydrogenase-2	10	0.023 ± .004	18	0.024 ± .003	3	0.083 ± .018
Mannose phosphate isomerase-2	10	0.801 ± .010	18	0.607 ± .009	1	0.630 ± .046
Muscle protein-1	10	0.000	18	0.063 ± .004	3	0.000
6-Phosphogluconate dehydrogenase	10	0.177 ± .009	18	0.266 ± .008	2	0.043 ± .018
Phosphoglucose isomerase	10	0.457 ± .012	18	0.479 ± .009	4	0.167 ± .023
Phosphoglucomutase-1	10	0.542 ± .012	18	0.589 ± .009	4	0.392 ± .031
Phosphoglucomutase-2	10	0.230 ± .011	18	0.324 ± .009	2	0.171 ± .047
Sorbitol dehydrogenase	7	0.211 ± .013	15	0.215 ± .008	1	0.000
Xanthine dehydrogenase	10	0.00	18	0.000	4	0.061 ± .015
	Monomorphic loci					
Acid phosphatase-1	7		18		1	
Adenylate kinase-2	10		18		1	
Esterase-2	10		18		1	
Hexokinase-1	10		18		2	
Malic enzyme (NADP) soluble	10		18		1	
Muscle protein-2	10		18		3	
Tetrazolium oxidase-1	10		18		1	
Tetrazolium oxidase-2	10		18		1	
Average heterozygosity (H_s)		0.224 ± .054		0.240 ± .055		0.117 ± .042

d is the number of subpopulations studied and H is the heterozygosity per locus ± S.E.

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EFFECTS OF BROWSING PREDATORS: ACTIVITY CHANGES IN INFAUNA FOLLOWING TISSUE LOSS

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ABSTRACT

Effects of tissue loss on defecation and/or tube building are documented for three infaunal species of polychaete annelids, *Abarenicola pacifica, Axiothella rubrocincta,* and *Spiophanes bombyx. Abarenicola* and *Axiothella* feed head down and expose their tails while defecating; their tail tips were experimentally ablated. *Spiophanes* feeds on the sediment surface with its pair of tentacles and its head. One or both of its tentacles were experimentally removed. The tissues removed in the experiments are those often lost to browsing predators in field populations. Defecation frequency and amount were significantly reduced in the experimental individuals relative to controls in all three species. In *Spiophanes* tube building was also significantly reduced; in *Axiothella* it was not. These results indicate that rates of biogenic sediment modification can be strongly affected by tissue losses of infauna to browsing predators.

INTRODUCTION

Tissue loss to browsing predators occurs commonly in plants (Harper, 1977; Grime, 1979) as well as animals. In marine sedimentary environments primary components of the diets of juvenile flatfish are the tails of arenicolid polychaetes and the siphons of tellinid bivalves (Macer, 1967; Edwards and Steele, 1968; Kuipers, 1973, 1977; deVlas, 1979a). In such habitats a significant percentage of the infauna preyed upon by browsing predators are usually regenerating body parts (Mangum, 1964; Ronan, 1975; deVlas, 1979b; Woodin, 1982). A variety of body parts are lost to such predators, especially those parts exposed to predatous risk above the sediment surface. Arenicolid and maldanid polychaetes, which usually live head downward and expose their tails to defecate, lose their tails (Mangum, 1964; deVlas, 1979b). Maldanids also lose their heads occasionally but whether this is due to a subsurface predator or to the maldanid exposing its head on the sediment surface is unknown (Wilson, 1979). Individuals which expose appendages or heads to feed are often found regenerating those parts (*e.g.,* sabellid polychaetes: Berrill, 1931; tellinid bivalves: Trevallion *et al.,* 1970; phoronid worms: Ronan, 1975; ophiuroids: Sides, 1981; spionid polychaetes: Woodin, 1982; venerid bivalves: Peterson and Quammen, 1982). Tissue loss appears to be a common occurrence at least for organisms which feed or defecate on the sediment surface.

In sedimentary habitats the infauna affect the survivorship and growth of one another by both indirect and direct interactions. The indirect interactions often occur via sediment modification (see reviews in Rhoads, 1974; Woodin and Jackson, 1979; Rhoads and Boyer, 1982; Thayer, 1983; Woodin, 1983). Defecation by organisms can modify sediments. The effects on the surrounding organisms are functions of the frequency of defecation and the amount per feculence (Brenchley, 1981). Any event that significantly affects the defecation rates will therefore indirectly affect growth and survivorship of other individuals.

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Given the apparent frequency of tissue loss, it is of interest to ask two questions. One, how do the activities of individuals suffering tissue loss differ from those of intact individuals? Two, how long is this activity difference maintained? This paper documents changes in activity resulting from tissue loss in three common polychaete families, the Arenicolidae, the Maldanidae, and the Spionidae, all of which frequently suffer tissue loss in the field.

MATERIALS AND METHODS

The following infaunal species were used in the experiments:

1. *Abarenicola pacifica* Healy and Wells, an arenicolid polychaete, lives head down in a J- or L-shaped burrow. It exposes its tail to defecate and leaves obvious spiral fecal castings on the sediment surface (Hobson, 1967). Size 54 to 75 mm (Hartman, 1969).
2. *Axiothella rubrocincta* (Johnson), a maldanid polychaete, lives head down in a vertical tube (see Kudenov, 1982 for a contrasting life style). It exposes its tail to defecate. Its feces are usually a fine layer spewed out onto the sediment surface. Size 70 to 120 mm but up to 200 mm (Hartman, 1969).
3. *Spiophanes bombyx* (Claparede), a spionid polychaete, lives head up in a tube. It exposes its pair of tentacles and sometimes its head to feed (Woodin, 1982). Its feces are deposited onto the sediment surface in long consolidated rods. Size 25 to 60 mm (Light, 1978).
4. *Pygospio elegans* Claparede, a spionid polychaete, lives head up in a tube. It exposes its pair of tentacles and head to feed and has fecal rods similar to those of *Spiophanes* (Woodin, 1982). Size 10 to 15 mm (Light, 1978).

In all of the experiments field collected animals were placed in cores of azoic sediment, and the activity rates of ablated individuals were compared to those of control individuals. For these experiments defecation and tube-building, both known to affect sediment properties, were measured (Table I). In all but one experiment one or more measurements were made of defecation activity and animals were monitored to determine when they resumed defecation. For species that build tubes, tubes in the sediment at termination were collected and weighed as an additional measure of activity (Table I).

All animals were used within 48 hours of collection. Damaged or regenerating individuals were rejected. The sediment for the experiments was collected from the same locale as the animals. The sediments were either treated with freshwater for 72 hours (*Abarenicola* experiments) or frozen for a minimum of 48 hours (all other experiments). The sediments were then flushed with sea water and sieved through a 0.5 mm mesh sieve in sea water to remove all large material. The cores were filled with sediment and placed in a sea water tank with running sea water for 24 hours before the experimental animals were added. Prior to addition of the animals, the tank was drained and refilled. Sediment samples for determination of organic carbon were taken at initiation and termination of each experiment. The samples were dried to constant weight at 80°C and then ashed at 500°C for four hours.

Abarenicola *experiments*

Abarenicola pacifica were collected in March 1979 from a muddy sandflat at False Bay, San Juan Island, Washington state (48°29'N:123°04'W). Individuals were cleaned of sediment and sorted into size groups. Individuals used in the experiment had a mean length of 3.2 cm (S.D. = 0.5, n = 80).

TABLE I

Summary of activity measurements for each experiment; all measurements were made in the laboratory unless otherwise indicated

1. Number of seconds from placing the individual on the sediment surface to complete burial was recorded at initiation of the experiment.
   Abarenicola (field and laboratory)
2. Individuals were monitored to determine when they resumed defecation after initiation of the experiment.
   Abarenicola (field and laboratory), *Axiothella, Spiophanes*
3. Individuals were observed for set time periods to determine number and/or amount of defecation(s) per time interval.
   Abarenicola (4 h) (weight and number of defecations)
   Axiothella (2 h) (area of core surface covered)
   Spiophanes (2 h) (weight and number of fecal rods)
4. Fresh feces were collected, dried, and weighed as a measure of fecal output per defecation.
5. Percent organic content of fresh feces was determined.
   Abarenicola
6. Percent organic content of sediment was determined at initiation and termination.
   Abarenicola (field and laboratory), *Axiothella, Spiophanes*
7. Depth of each individual's burrow was measured at termination.
   Abarenicola (field and laboratory)
8. Tube material in the cores was collected and dried and weighed at termination.
   Axiothella, Spiophanes, Pygospio

Animals were assigned by random number to treatments (control, experimental), locale (field, laboratory), block within locale, and duration type (13 or 25 days). There were 20 individuals per treatment per locale per duration type, a total of 160 individuals. The tips of the tails of the experimental individuals were ablated with a scalpel. Less than 5 mm was removed in all cases. The individuals were placed on the surfaces of their assigned cores in sea water and their burrowing times recorded. The experimental containers in the laboratory experiments were aged 1000 ml plastic beakers (11.3 cm diam. and 14.3 cm tall) filled to the rim with sediment. In the field the containers were of equal dimensions but of fiberglass windowscreen (1 mm mesh) glued together with Hot Melt Glue (Thermogrip R). Prior to transportation of the mesh cores to the field, they were wrapped with plastic to prevent loss of sediment. The plastic wrap was removed prior to implantation. Twenty-four hours after initiation the field cores were implanted in the area from which the animals had originated. A sediment core of the same size as the mesh experimental core was removed. An external sleeve held the surrounding sediment in place while the experimental core was inserted and then the sleeve was removed. The mesh edge of the experimental core protruded 1 to 5 mm above the sediment surface. Both in the field and in the laboratory the cores were arranged randomly within blocks.

The laboratory and the field cores were monitored daily for the presence of fecal castings. On days 6, 12, 18, and 24 after initiation the laboratory cores were cleared of surface feces by waving a spoon in the water above the sediment. The cores were then observed for 4 hours, and all feces were collected as they were deposited by carefully picking up the mucous-bound fecal matter with a fork and a spoon. The time of deposition of the feces was recorded. The feces were dried and ashed to determine organic content.

Both in the field and in the laboratory half of the cores were terminated on day 13 and half on day 25. At termination sediment samples were taken from a. the sediment surface, b. the head shaft of the animal's burrow, and c. the sediment adjacent to, but not part of, the head shaft. In the field cores these samples were taken in the field immediately after removing the core from the surrounding sediment. The depth of the burrow and the condition of the individual were noted.

Axiothella *experiments*

Axiothella rubrocincta were collected in July 1982 from the mid-intertidal zone of a sand flat at False Bay. They were removed from their tubes, cleaned, and assigned by random number to a treatment (control, experimental). The tip of the tail of each experimental individual was ablated (pygidium plus one or two segments, less than 5 mm). The individuals were placed on the surface of their assigned cores and timed to complete burial.

The experimental cores were 15 cm lengths of 8 cm internal diameter PVC pipe which had been aged in sea water prior to the experiment. The cores were filled with sediment to within 1 cm of the rim of the core and placed in a Latin square design in a running sea water table. There were 10 replicates per treatment for a total of 20 cores.

The cores were monitored daily for evidence of feces. At least every other day the core surface was cleared of feces. A layer of calcium carbonate powder (less than 1 mm thick) was spread over the surface with a double screen flour duster. This provided a white background against which fresh feces could be photographed. The core was checked after one hour for feces. At two hours the cores were individually photographed using a camera with a macrolens and two strobes. The tank was drained prior to photographing to reduce reflections. The area of the fecal material on the core surface was determined from the negative with a digitizer interfaced with a computer. Such fecal areas are significantly correlated with fecal weights and volumes (MacRae, 1983). At termination a sediment sample was taken from the surface of each core for organic content analysis. Each animal was removed from its core and the sediment was sieved on a 1 mm mesh sieve to remove all tube material. The tubes were dried and weighed.

Spiophanes *experiments*

Individuals of *Spiophanes bombyx* were collected in April 1982 from an intertidal muddy sand habitat on Debidue Creek, North Inlet, Georgetown, South Carolina (33°20'N:79°10'E). Undamaged individuals were assigned by random number to a treatment (control, missing one tentacle, missing two tentacles) and a block (5). Either one or both tentacles were removed by pulling lightly on the tentacle. The individuals were then placed on the surface of their assigned cores. There were two replicates per treatment per block for a total of 30 cores. The cores were 2.8 cm diam. by 11.0 cm high plastic centrifuge tubes that had been aged in sea water. They were arranged in a randomized block design in a 36 cm by 31 cm by 18 cm deep tank in running sea water. After 12 hours the cores were transported 125 miles to Columbia, South Carolina where they were kept in the same tank in aerated sea water in an incubator at 16°C on a 12 hour day-night cycle.

Every day the core surfaces were cleared of all fecal rods. Two hours after clearing the new fecal rods were collected from the surface with a pipette, counted, dried, and weighed. At termination (13 days) a surface sediment sample was taken for organic analysis and then the core sediments were sieved on a 0.5-mm mesh sieve. The tubes were collected, dried, and weighed.

Pygospio *experiments*

Individuals of *Pygospio elegans* were collected in July 1982 from an intertidal muddy sand habitat in False Bay. Individuals were assigned by random number to a treatment (control, missing one tentacle, missing two tentacles) and a block (5). There were two replicates per treatment per block for a total of 30 cores. After assignment depending upon the treatment one or both tentacles of experimental individuals were removed by lightly pulling on the tentacle. The individual was then placed on the surface of its assigned core. The core was 2.8 cm diam. by 11.0 cm high plastic centrifuge tube that had been aged in sea water. They were arranged in a randomized block design in running sea water in a 20 cm deep sea water table. At termination (seven days) the core sediments were sieved on a 0.5 mm mesh sieve. The tubes were collected, dried, and weighed.

Statistical analyses

In all experiments except those with *Pygospio* fecal samples were taken over time from the same cores (see Table I). These were analyzed using a repeated measures analysis of variance (Winer, 1971). Several of the analyses involved nested ANOVA's (Winer, 1971; Kirk, 1982). These cases are indicated in the Results. When the data did not involve repeated measures or nested designs, a standard analysis of variance was used. When significant effects ($P < 0.05$) or strong trends ($0.05 < P < 0.10$) were indicated by the results of the analysis of variance, and the interaction terms were not significant, a Bonferroni test was used to identify differences among the treatments (Neter and Wasserman, 1974). The experimentwise error rate was 0.05. Either a Chi-square Test or a Fisher's exact test was used to compare the rates of return to defecation of control and ablated individuals of *Abarenicola, Axiothella,* and *Spiophanes* (Siegel, 1956).

RESULTS

Abarenicola *experiments*

Burrowing times measured at initiation did not differ significantly among individuals assigned to the field and the laboratory experiments (nested ANOVA, locale, F = 0.01, df = 1, 2, $P > 0.91$). Burrowing times were also not significantly different between ablated and control individuals (nested ANOVA, treatment, F = 0.03, df = 1, 2, $P > 0.87$) nor was the interaction term of treatment and locale significant (nested ANOVA, F = 1.14, df = 1, 2, $P > .39$). Burrowing times in seconds (means and standard deviations) were as follows: field: control 119.4 (49.9), exp. 130.6 (80.4); laboratory: control 126.6 (36.4), exp. 119.1 (42.4).

As predicted, ablated individuals were slower to resume defecation than control individuals in the field as well as in the laboratory (Fig. 1). In the field a majority of ablated individuals resumed defecation within six days while in the laboratory a majority returned within eight days. Although experimental individuals in the laboratory are slower to resume defecation than those in the field, the pattern is similar (Fig. 1) suggesting that the measurements confined to laboratory populations are informative (see Table I for a summary of laboratory and field measurements). In the laboratory the proportion of control individuals defecating within the four-hour observation period was significantly greater than the proportion of experimental individuals defecating (Table II, Active) (Chi-square: 13 day: $\chi^2 = 11.66$, df = 1, $P < 0.001$; 25 day: $\chi^2 = 42.13$, df = 1, $P < 0.0001$).

In the laboratory within a four-hour period of observation ablated individuals

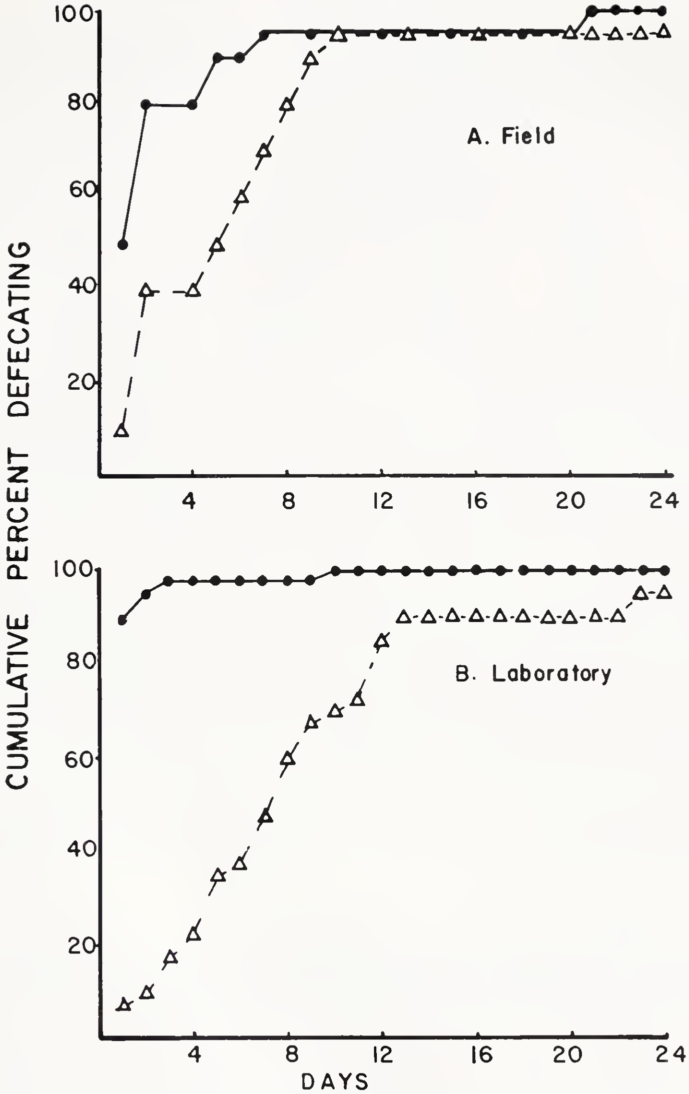

FIGURE 1. Cumulative percentage of *Abarenicola* defecating in cores in the field (A) and in the laboratory (B). Means and standard deviations of control (●) and experimental individuals (△). Days are number of days since initiation of the experiment.

defecated significantly less frequently than did the controls (Table II) (repeated measures ANOVA with individuals nested under cross of treatment and block, 13 day exp: F = 5.55, df = 1, 36, $P < 0.025$; 25 day exp.: F = 15.44, df = 1, 36, $P < 0.0001$). The number of defecations observed changed with time (significant date effect) (13 day exp.: F = 4.37, df = 1, 36, $P < 0.05$; 25 day exp.: F = 4.52, df = 3, 108, $P < 0.005$) but not as a function of treatment (date ∗ treatment interaction effect, 13 day exp.: F = 1.65, df = 1, 36, $P > 0.2$; 25 day exp.: F = 1.73, df = 3, 108, $P > 0.16$). The lack of a significant date ∗ treatment interaction term indicates that the significant treatment difference persists throughout the experiment (25 days). The

TABLE II

Abarenicola *experiment: number of defecations, fecal weights, and proportion of individuals defecating during four-hour periods of observation in the laboratory*

		No. def.		Fecal wt.		Active	
	Day	C	E	C	E	C	E
A.	6	1.0 (1.8)	0.2 (0.9)	.1427 (.1773)	.0098 (.0303)	.45	.10
	12	3.3 (5.0)	0.7 (3.4)	.1147 (.1371)	.0051 (.0230)	.45	.05
B.	6	2.3 (3.9)	0.5 (1.6)	.1401 (.1518)	.0232 (.0609)	.50	.15
	12	6.1 (4.8)	1.2 (3.9)	.1994 (.1460)	.0199 (.0636)	.70	.10
	18	3.6 (4.2)	0.7 (2.3)	.1568 (.1440)	.0198 (.0618)	.65	.10
	24	4.9 (4.9)	1.6 (3.6)	.1682 (.1486)	.0441 (.0966)	.70	.20

Means and standard deviations. n = 20 worms per treatment in each experiment. A. Experiment terminated on day 13. B. Experiment terminated on day 25. No. def. = number of defecations per worm per 4 hours. Fecal wt. = mean weight in grams of feces produced per worm per defecation during the 4-hour period. Active = proportion defecating within 4-hour period. C = control individuals. E = experimental individuals (ablated tail tips). Day = number of days elapsed since initiation.

ablated individuals had not yet recovered 25 days after ablation. Although the majority of the ablated individuals resumed defecation within six to eight days, frequency and amount per defecation are still below those for controls at 24 days (Table II).

The results for fecal weights are similar to those for number of defecations (Table II). The weights of individual fecal piles were significantly lower for ablated individuals than for control individuals (Table II) (repeated measures ANOVA with individuals nested under cross of treatment and block, 13 day exp.: F = 13.24, df = 1, 36, $P < 0.001$; 25 day exp.: F = 34.34, df = 1, 36, $P < 0.0001$). There were no significant differences among dates nor was the date ∗ treatment interaction significant indicating that the treatment effect did not disappear by day 12 or 24 (date effect: 13 day exp.:

TABLE III

Abarenicola *experiment: percent organic content of sediments*

		Locale	Control	Experimental
A.	Surface			
		Field	1.3 (0.2, 13)	1.2 (0.3, 14)
		Lab	1.7 (0.2, 12)	1.8 (0.6, 17)
	Feeding area			
		Field	1.4 (0.2, 12)	1.6 (0.3, 10)
		Lab	1.7 (0.4, 15)	1.4 (0.2, 17)
	Below sediment			
		Field	1.4 (0.1, 14)	1.5 (0.3, 13)
		Lab	1.6 (0.3, 15)	1.4 (0.3, 15)
B.	Feces	Lab		
		Day 12	1.6 (0.2, 14)	1.6 (0.4, 3)
		Day 24	1.6 (0.5, 27)	1.6 (0.2, 10)

Means and standard deviations and number of replicates. Differences between controls and experimentals are not significant; see text. A. Sediments collected at termination from the core surfaces, adjacent to the head of the worm (feeding area) and at burrow depth but not adjacent to the burrow (below sediment). B. Fresh feces collected on days 12 and 24.

TABLE IV

Abarenicola *experiment: ANOVA table for percent organic content of sediments from core surfaces, the sediments adjacent to the head of the worm, and the sediments at burrow depth but not adjacent to the burrow (see Table IIIA)*

	df	MS	F	*P*
Treatment	1	5.34	0.495	.483
Sample type	2	9.40	0.871	.420
Locale	1	198.24	18.374	.0001
Treatment × locale	1	36.33	3.367	.068
Treatment × sample	2	4.84	0.448	.640
Locale × sample	2	97.79	9.064	.0001
Treatment × locale × sample	2	41.51	3.847	.023
Error	155	10.79		

F = 1.07, df = 1, 36, $P > 0.3$; 25 day exp. F = 0.83, df = 3, 108, $P > 0.45$) (date * treatment interaction: 13 day exp.: F = 0.55, df = 1, 36, $P > 0.45$; 25 day exp.: F = 0.88, df = 3, 108, $P > 0.45$). The percent organic content of the feces was not significantly different between ablated and control groups (ANOVA, treatment, F = 0.24, df = 1, 58, $P > 0.62$) nor was there a significant date effect (ANOVA, date, F = 1.93, df = 2, 58, $P > 0.15$) (Table IIIB).

The percent organic contents of the sediment surface, the feeding area, and the sediment at burrow depth were not significantly different from one another at termination (Tables III and IV, sample type not significant). There was no significant difference between ablated and control treatments (Tables III and IV, treatment not significant). There was however a significant locale effect as well as significant locale * sample and locale * sample * treatment interactions (Table IV). Depths of burrows at termination (25 days) also showed a significant locale effect but not a significant treatment effect (Table VB). Field burrows were significantly shallower than burrows made by individuals in the laboratory regardless of treatment (Table VA).

Axiothella *experiments*

The proportion of individuals defecating within the two-hour period of observation differed between the controls and the experimentals up through day six (Table VI)

TABLE V

Abarenicola *experiment: depth in cm of burrow at termination*

A.	Control	Experiment
Field	10.75 (0.84, 18)	10.03 (1.31, 16)
Laboratory	12.04 (0.41, 20)	12.18 (0.35, 19)

B.	df	MS	F	*P*
Treatment	1	1.34	6.25	.130
Treatment × locale	1	3.13	14.56	.062
Error	2	0.21		
Locale	1	52.39	125.98	.008
Block within locale (Locale error)	2	0.42	0.64	.529
Within cell error	65	0.65		

A. Means and standard deviations and number of replicates. B. ANOVA table for nested design with block nested under locale and locale crossed with treatment.

TABLE VI

Axiothella *experiment: proportion of individuals that defecated during two hour observation period*

Day	Control	Experimental
1	0.00	0.00
2	.80	.60
4	.90	.50
6	.70	.40
8	1.00	1.00
10	1.00	1.00
12	.90	1.00
15	.90	1.00

Number of replicates is ten per treatment for all dates. All worms in both treatments had defecated by day 4. Ninety percent of the controls and one hundred percent of the experimentals had defected by day two. Experimental individuals had their tails ablated.

(Chi-square, days 1–6, $\chi^2 = 4.05$, df = 1, $P < 0.05$; Fisher's Exact test, days 8–15, $P > 0.2$). The proportion of the sediment surface covered by feces within the two hours differed significantly among treatments and dates (Fig. 2, Table VII). The treatment ∗ date interaction was also significant (Table VII). As is true in other maldanids (Dobbs, 1983) the activity rates of the controls as well as those of the experimental individuals appear to show a reaction to handling (Fig. 2). However the controls show significantly greater defecation activity than the experimental individuals (Fig. 2).

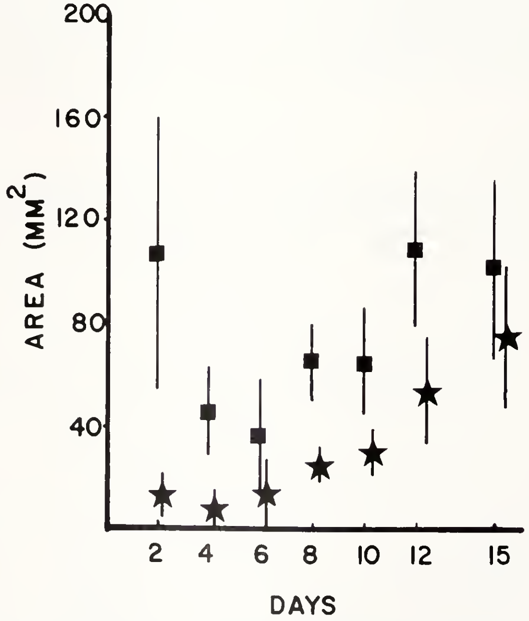

FIGURE 2. *Axiothella* experiment. Area of core surface in mm^2 (core area 5027 mm^2) covered by feces after two hours. Ten replicates per treatment. Means and 95% confidence intervals of control (■) and experimental individuals (★). Days are number of days since initiation of the experiment.

TABLE VII

Axiothella *experiment: ANOVA table for surface area of core covered by feces after two hours*

	df	MS	F	*P*
Treatment	1	69154.02	24.50	.0001
Error	18	2823.05		
Date	6	12038.31	9.57	.00001
Treatment × date	6	2976.45	2.37	.0346
Error	108	1257.44		

Repeated measures design, cores nested under treatments. Ten replicates per treatment. See Figure 2.

At termination the weights of tubes collected from the cores did not differ significantly between treatments (ANOVA, $F = 0.739$, $df = 1, 18$, $P > 0.40$) although the mean tube weight collected in the experimental cores was less than that collected from the control cores (Table VIII). Percent organic content of the surface sediments also did not differ between treatments at termination (ANOVA, $F = 0.16$, $df = 1, 18$, $P > 0.69$) [means and standard deviations, controls: 1.1 (0.1), exp.: 1.1 (0.1)].

Spiophanes *experiments*

The proportion of individuals defecating during the two-hour observation period did not differ significantly between the controls and individuals missing one tentacle (Chi-square analysis, $\chi^2 = 0.894$, $df = 1$, $P > 0.34$) (Table IXA). The proportion of control individuals defecating was significantly different from the proportion defecating of the individuals missing two tentacles (Chi-square analysis, $\chi^2 = 15.701$, $df = 1$, $P < 0.0001$) (Table IXA). The proportion defecating of the individuals missing two tentacles was also significantly different from the proportion defecating of the individuals missing one tentacle (Chi-square analysis, $\chi^2 = 7.427$, $df = 1$, $P < 0.006$) (Table IXA). In both cases the proportion defecating of the individuals missing two tentacles was significantly lower than those for the controls or the individuals missing one tentacle (Table IXA). These differences persisted throughout at least the first seven days of the experiment (Table IXA). In all treatments the majority of the individuals resumed defecation within two days (Table IXB).

The total weight of feces produced during a two hour period differed significantly among treatments and among dates (Table X) (repeated measures ANOVA, treatment, $F = 6.27$, $df = 2, 8$, $P < 0.03$; date, $F = 3.37$, $df = 6, 48$, $P < 0.008$). The date *

TABLE VIII

Weights in grams of tubes collected at termination of the experiments

	Control	Experimental	
A.	3.072 (1.068)	2.638 (1.186)	
	Control	Missing One Tentacle	Missing Two Tentacles
B.	.1155 (.0271) a	.0835 (.0351) c	.0972 (.0241) a c
C.	.0079 (.0019) a	.0077 (.0047) a	.0068 (.0016) a

Means and standard deviations. A. *Axiothella* tubes. Ten replicates per treatment. Difference between means is not significant; see text. B. *Spiophanes* tubes. Ten replicates for control and missing two tentacle treatments, seven for missing one tentacle treatment. C. *Pygospio* tubes. Ten replicates per treatment. Differences in letters between values in a row indicate significant differences (Bonferroni *t*-test).

TABLE IX

Spiophanes *experiment: A. proportion of individuals which defecated during the two hour period of observation; B. cumulative proportion defecating*

	Day	C	1T	2T
A.	2	.90	.86	.50
	3	.70	.71	.60
	4	1.00	1.00	.50
	7	1.00	.86	.50
	9	1.00	1.00	.80
	11	1.00	.86	.80
	13	.90	.86	.90
B.	1	1.00	.60	.30
	2	1.00	1.00	.90
	3	1.00	1.00	1.00

C = control individuals, 1T = individuals missing one tentacle, 2T = individuals missing two tentacles.

treatment interaction term was not significant indicating that the treatment differences persisted throughout the 13 days of the experiment (repeated measures ANOVA, F = 0.84, df = 12, 48, $P > 0.61$). Bonferroni *t*-tests revealed that the fecal weights for the individuals missing two tentacles were significantly different from those of the control and the individuals missing one tentacle but that the values for the individuals missing one tentacle were not significantly different from those of the

TABLE X

Spiophanes *experiment: total weight of feces (grams) produced during two-hour periods by single individuals*

Day	C	1T	2T	Date Means
2	.0017 (.0012, 10)	.0010 (.0006, 7)	.0006 (.0010, 10)	.0011 a
3	.0014 (.0011, 10)	.0010 (.0009, 7)	.0010 (.0010, 10)	.0013 a
4	.0016 (.0007, 10)	.0018 (.0008, 7)	.0008 (.0010, 10)	.0012 a
7	.0019 (.0005, 10)	.0015 (.0008, 7)	.0008 (.0009, 10)	.0013 a d
9	.0017 (.0003, 10)	.0018 (.0007, 7)	.0013 (.0008, 10)	.0016 a d e
11	.0022 (.0004, 10)	.0018 (.0009, 7)	.0014 (.0008, 10)	.0018 b d e
13	.0017 (.0008, 7)	.0024 (.0009, 6)	.0018 (.0008, 10)	.0019 b e
Treatment Means	.0017 a	.0016 a	.0011 b	

C = control individuals, 1T = missing one tentacle individuals, 2T = missing two tentacle individuals. Means and standard deviations and number of replicates per date. Factor level means of treatments and dates. Differences in lower case letters indicate significant differences among factor level means (Bonferroni *t*-test).

controls (Table X). Mean fecal weights for days 2 to 4 were significantly different from those for days 11 and 13. Days seven and nine were intermediate (Table X).

The number of fecal rods produced during a two-hour period also differed significantly among treatments and among dates (Table XI) (repeated measures ANOVA, treatment, F = 4.83, df = 2, 8, $P < 0.05$; date, F = 3.24, df = 6, 48, $P < 0.01$). As for total fecal weight the date * treatment interaction term was not significant indicating that the treatment differences persisted throughout the 13 days of the experiment (repeated measures ANOVA: F = 1.53, df = 12, 48, $P > 0.14$). Bonferroni *t*-tests revealed that the mean numbers of fecal rods differed significantly between the control individuals and the individuals missing two tentacles. Values for individuals missing one tentacle, however, were not significantly different from those for individuals missing two tentacles or from values for control individuals (Table XI). Mean numbers of fecal rods for days 2 through 7 were significantly different from those for days 9, 11, and 13 (Table XI).

At termination the tubes in the cores were collected, dried, and weighed. The ANOVA revealed no significant differences at the 0.05 level although the treatment effect had a probability of 0.061 (Table XII). A Bonferroni *t*-test showed that the control cores had significantly greater total tube weights than the cores with individuals missing one tentacle (Table VIIIB). Total tube weights were not significantly different between cores with control individuals and cores with individuals missing two tentacles. Cores with individuals missing two tentacles were also not significantly different from those with individuals missing one tentacle (Table VIIIB). At termination percent

TABLE XI

Spiophanes *experiment: total number of fecal rods produced during two-hour periods by single individuals*

Day	C	1T	2T	Date Means
2	3.0 (2.2, 10)	2.4 (2.6, 7)	1.1 (1.4, 10)	2.1 a
3	2.2 (1.8, 10)	1.1 (1.3, 7)	1.5 (1.5, 10)	1.8 a
4	3.0 (1.8, 10)	2.7 (1.0, 7)	1.0 (1.4, 10)	2.0 a
7	3.6 (1.6, 10)	2.3 (1.6, 7)	1.2 (1.3, 10)	2.1 a
9	3.1 (1.7, 10)	2.3 (2.2, 7)	3.1 (2.6, 10)	3.1 b
11	4.4 (1.6, 10)	3.1 (1.9, 7)	2.5 (1.6, 10)	3.4 b
13	2.7 (2.0, 7)	4.2 (2.6, 6)	3.7 (2.6, 10)	3.5 b
Treatment Means	3.2 a	2.6 a b	2.0 b	

C = control individuals, 1T = individuals missing one tentacle, 2T = individuals missing two tentacles. Means and standard deviations and number of replicates per treatment per date. Factor level means of treatments and dates. Differences in letters indicate significant differences among factor level means (Bonferroni *t*-test).

Table XII

Spiophanes *experiment: ANOVA table for weight of tubes*

	df	MS	F	*P*
Treatment	2	2039.87	3.56	.061
Block	4	769.48	1.34	.310
Treatment × Block	8	1235.42	2.15	.111
Error	12	573.19		

Two-way ANOVA with treatment crossed with block. See Table VIII.

organic content of surface sediments did not differ significantly among treatments (ANOVA, F = 2.66, df = 2, 24, $P > 0.09$).

Pygospio *experiments*

Pygospio is smaller than *Spiophanes* so no attempt was made to measure activity other than by weight of tubes collected at termination. Tube weights did not differ significantly among the treatments (Table VIIIC) (ANOVA, F = 0.231, df = 2, 12, $P > 0.50$).

Discussion

Biogenic alteration of sediments is known to affect the flux of compounds into and out of interstitial water (Aller, 1978, 1982; McCaffrey *et al.*, 1980; Waslenchuk *et al.*, 1983), the ability of sediments to be resuspended (Rhoads and Young, 1970; Rhoads, 1974; Myers, 1977a, b; Rhoads *et al.*, 1978) and the physical structure of the sediment (Rhoads, 1974; Myers, 1977a; Rhoads and Boyer, 1982). Indirect interactions among infauna often are mediated by such biogenic alterations of the sediment. The concept of 'trophic amensalism' (Rhoads and Young, 1970) and its subsequent modifications (Woodin, 1976; Brenchley, 1981) are descriptions of how biogenic alteration of sediments by the movements and feeding and defecation of infauna affects the survivorship and/or emigration of infauna (Weinberg, 1979; Brenchley, 1981, 1982; Wilson, 1981) as well as the growth rates of individuals (Rhoads and Young, 1970).

The rate at which infauna modify the sediments is usually described as being a function of their species, size, and density as well as physical variables such as temperature (Mangum, 1964; Rhoads, 1967, 1974; Cadee, 1976; Kudenov, 1982; Dobbs, 1983; Thayer, 1983). It may also be a function of their health as that affects their activity. Primary dietary components of juvenile flatfish often are the tails and tentacles of worms and the siphons of bivalves (Macer, 1967; Trevallion *et al.*, 1970; Braber and deGroot, 1973; Kuipers, 1977; deVlas, 1979a); regenerating worms and bivalves are common in infaunal samples (Mangum, 1964; deVlas, 1979b; Wilson, 1979).

The results of these experiments demonstrate that tissue loss affects the activities of three polychaetes, *Abarenicola, Axiothella,* and *Spiophanes.* Defecation frequency and fecal amounts were negatively affected in all three species. Ablated individuals of *Abarenicola* were significantly slower to return to defecation after the initiation of the experiment than controls (Fig. 1); they had significantly fewer defecations per time interval than controls (Table II), and their fecal piles were significantly lighter in weight than those of control individuals (Table II). In *Axiothella* the proportion of control individuals defecating was larger than that of the experimental individuals

(Table VI) and the amount of the core surface covered by feces was significantly greater in the control cores (Table VII, Fig. 2). In *Spiophanes* loss of both tentacles significantly affected the proportion of individuals defecating, the total weight of feces, and the total number of fecal rods (Table IX, X, and XI); loss of one tentacle did not significantly affect these parameters. Loss of one tentacle did significantly affect the weight of tubes produced (Table VIIIB).

Some measurements were not significantly affected by tissue loss. In none of the experiments was percent organic content of sediments or feces affected (Tables III and IV). Ablated individuals of *Abarenicola* were not significantly different from control individuals in burrowing time at initiation and depth of burrow at termination (Table V). In the experiments with *Axiothella* and *Pygospio* total tubes collected at termination did not differ significantly in weight between ablated and control individuals (Table VIIIA and VIIIC).

If organisms have important influences on the chemical alteration of sediments (Aller, 1978, 1982), the physical structure of the sediments (Rhoads, 1974), and the rate of resuspension of sediments (Rhoads and Young, 1970), then any process that changes the rate at which sediment modification occurs is of interest. Tube building and defecation are two of the activities of infauna that affect sediment properties (Myers, 1977a, b; Rhoads *et al.*, 1978; Eckman, 1979, 1983; Eckman *et al.*, 1981). These experiments indicate that small amounts of tissue loss affect the rates of these activities. Both the literature on the contents of fish guts (Edwards and Steele, 1968; Braber and deGroot, 1973; Kuipers, 1977; deVlas, 1979a) and the data from static samples of infauna (Mangum, 1964; Ronan, 1975; Wilson, 1979; Woodin, 1982) indicate that such losses are common in natural populations. Models of biogenic alteration of sediments need to consider the implications of the period of relative inactivity of individuals suffering tissue losses. Rates of sediment modification measured on intact animals in the laboratory may grossly overestimate activities in field populations exposed to browsing predators.

The three polychaete species used in this study belong to three families that are very common in the intertidal zone and along the continental shelves of the Atlantic and Pacific Oceans. Arenicolids are often the most common large infaunal organism in samples from shallow water on the European coast (Beukema and deVlas, 1979) as well as elsewhere (Hobson, 1967). Both arenicolids and maldanids increase the rate at which sediment turns over (Hobson, 1967; Rhoads, 1967; Cadee, 1976; deVlas, 1979b). Both also appear to affect the local composition of the infauna through their effect on the sediment (Wilson, 1981; Weinberg, 1979). Most members of both families feed at depth and defecate on the surface. For these organisms it is this defecation onto the sediment surface that alters the local sediment turnover rates and affects the rest of the infauna. Spionids, although much smaller, are very common and are known to change the stability of sediments by their tube-building activities (Featherstone and Risk, 1977). The tube-building activities of maldanids can also significantly affect sediment properties (Rhoads, 1974; Featherstone and Risk, 1977). The results of these experiments indicate that small amounts of tissue loss will dramatically alter rates of sediment alteration. Clearly, in attempting to assess the importance of biogenic sedimentary change in determining the composition of the local assemblage, as well as the chemical and physical structure of the sediment, it is necessary to know the activity rates of the individuals determining biogenic sediment turnover rates. If the rates of recovery from tissue loss indicated in this paper are generally true, sediment turnover rates calculated from laboratory and/or field observations on intact individuals may be incorrect by an order of magnitude or more depending upon the frequency of tissue loss in the field.

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COLLECTION OF FOOD BY ORAL SURFACE PODIA IN THE SAND DOLLAR, *ECHINARACHNIUS PARMA* (LAMARCK)

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ABSTRACT

In *Echinarachnius parma* all spine types have cilia arranged in two bands along the shaft. Ciliary currents flow perpendicular to these bands and reversals were not observed. On the aboral surface the bands of cilia were oriented perpendicular to lines radiating from the apex. Flow visualization using dyes and particles showed that aboral currents flow radially towards the ambitus. In contrast, on the oral surface, currents flow from anterior to posterior and the bands of cilia are arranged at right angles to this axis. Oral surface ciliary currents do not carry particles to the mouth nor to the food grooves. At least 80% of the particles carried over the aboral surface are lost at the ambitus. Only particles $< 20\ \mu$m, if any, pass around the ambitus and these were not seen to enter the food grooves.

Light microscope observations showed that oral surface and ambital podia continuously probe the substrate and draw particles towards the test. Particles are passed from podium to podium, to the food grooves, and thence to the mouth. The rate of particle collection and passage along the food grooves is much higher when concentrated diatoms are offered than with normal sand substrate. This oral surface activity has not been reported before in any sand dollar.

Echinarachnius parma discriminates against substrate particles larger than 230 μm in diameter, but does not especially select those less than 100 μm. Statistical analysis showed significantly fewer particles $> 230\ \mu$m in the gut, when compared to natural sediment ($P < 0.05$). However, there was no significant difference in proportions of smaller particles. Thus there is no evidence that these sand dollars used an aboral sieving mechanism which would have concentrated particles $< 100\ \mu$m from the substrate.

INTRODUCTION

Sand dollars (Clypeasteroida) are flat, irregular echinoids with a lantern apparatus. They live on soft substrates, mostly in shallow water. *Echinarachnius parma* (Lamarck) occurs in the north Atlantic and Pacific on mixed sandy substrates (Harold and Telford, 1982), where it extends from the intertidal zone to depths of about 1600 m (Mortensen, 1948). All sand dollars are adapted to collect particulate food from the substrate material and also to maintain their position on or in the bed of mobile sediment in moving water. In the recent literature a dichotomy of opinion has emerged between those who interpret sand dollar morphology primarily in terms of feeding (Ghiold, 1979; Seilacher, 1979; Alexander and Ghiold, 1980; Smith and Ghiold, 1982; *inter alia*) and those who give primacy to hydrodynamic forces (O'Neill, 1978; Telford, 1981, 1983; Telford and Harold, 1982).

Goodbody (1960) was the first investigator to provide a detailed description of feeding in any sand dollar [*Leodia sexiesperforata* (Leske)]. According to his observations, substrate material was passed over the aboral surface, supported on the club-

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shaped spines. Small organic and inorganic particles which fell between the spines were swept to the ambitus in ciliary currents, transferred to the oral surface, and then swept into the food grooves for transport to the mouth. This explanation of feeding has been very widely accepted and has provided the basis for later descriptions of feeding in *Mellita quinquiesperforata* (Leske) (Ghiold, 1979; Alexander and Ghiold, 1980; Lane and Lawrence, 1982) and in *E. parma* (Mooi and Telford, 1982; Ghiold, 1983). Seilacher (1979) coined the descriptive term "rocking sieve" for this mechanism and treats sand dollars as little mobile sieves. In his opinion sand dollars had deep-burrowing ancestors and their modern flattened form is an adaptation to sifting surface sediments while burrowing to shallow depths.

In contrast to this conventional view, Telford (1981, 1983) has argued that sand dollars are specially adapted for life in moving water on unstable substrates and, by implication, they might well have had a non-burrowing ancestry. The flattened form is seen as an adaptation reducing the drag profile; the lunulate sand dollars (Mellitidae, Astriclypeidae, and Rotulidae) are further modified to reduce lift; shallow burrowing is a behavioral adaptation which effectively places the organism low in the boundary layer or entirely beneath it. These ideas do not contradict the notion of feeding by sieving the sediment. However, that mechanism is not essential in the hydrodynamic interpretation of form, whereas it is the very basis of interpretation for adherents to the rocking sieve hypothesis.

Feeding mechanisms need to be critially re-evaluated in light of these different explanations of sand dollar morphology. The first suggestion of the ciliary-mucus model [for *Dendraster excentricus* (Eschscholtz)] was given by McGinitie and McGinitie (1949), with minimal supporting data. Chia (1969) accepted this view but Timko (1976) re-examined feeding in *D. excentricus* and rigorously demonstrated that it collects particles by use of spine-cone traps, pedicellariae, and podia. Later, O'Neill (1978) proposed a mechanism for the exploitation of hydrodynamic forces in group facilitation of feeding in this species. To date, Timko's (1976) work has contained the only suggestion of food collection by oral surface podia in a sand dollar. All investigators have remarked on substrate probing by podia and on the activity of ambital and aboral podia in drawing particles onto the spine canopy, which has been reaffirmed by Ghiold (1983). In spite of this, Ghiold (1979, 1983) and Lane and Lawrence (1982) rejected any involvement of podia on the oral surface during food collection. Seilacher (1979) mentioned the activity of oral surface spines in stirring the sediment and allowed the possibility of podial collection of particles. More recently, Telford *et al.* (1983) have shown that *Echinocyamus pusillus* (O. F. Muller) (Fibulariidae) relies entirely on the podia for collection and transportation of food particles. In several investigations, analysis of sand dollar gut contents has demonstrated the ingestion of particles considerably larger than could be accommodated by the sieving mechanism (Chia, 1969; Ghiold, 1979, 1983; Mooi and Telford, 1982; Lane and Lawrence, 1982). These results have remained unexplained or have been dismissed as accidental.

The present study was undertaken to examine and quantify the possible contribution of the oral surface to feeding in *Echinarachnius parma.* This eventually included a complete re-examination of the rocking sieve mechanism, the ciliary transport of particles, and their passage around the ambitus.

MATERIALS AND METHODS

Echinarachnius parma was collected from the intertidal zone of Bar Road, a natural causeway near St. Andrews, New Brunswick, in August 1982.

The aboral surface of live sand dollars was observed from above using a conventional dissecting microscope; the oral surface from below using a single-plane mirror; and the ambitus from the side using a boom-mounted dissecting microscope (Mooi and Telford, 1982). Ciliary currents were made visible by several means, including carmine particles, yeast, milk, diatoms, or small sand grains pipetted onto the sand dollar. The direction of flow was mapped on oral and aboral surfaces as well as around the ambitus. Observations of podial activity were made using natural substrate from the sand dollar bed, concentrated planktonic diatoms, filamentous and adherent pennate diatoms, hydrated *Artemia* eggs and nauplii, and carmine particles. These experimental materials, alone or in various combinations, were pipetted onto the aboral surface, around the ambitus, or introduced beneath the animals for oral surface observations.

Specimens were relaxed with ethanol (Mooi, 1983) and fixed for SEM in 2% glutaraldehyde for 24 hours, then transferred to 2% buffered formalin for storage. Small pieces of test with attached spines were critical point dried and sputter coated with gold before examination with a Cambridge 180 SEM. Additional observations of spines and podia were made by light microscope.

Inorganic particle size-frequency distribution in gut contents was compared to that of natural substrate. Sand dollars rapidly empty the digestive system during live dissection (Hyman, 1958; *inter alia*). Bell and Frey (1969) found that guts remained full if specimens were fixed immediately after collection. Accordingly, for this purpose, 15 sand dollars were collected from Bar Road and immediately preserved in 10% formalin. They were all collected from a small area of the beach, less than 4 m apart. Directly adjacent to each sand dollar a 1 cm deep substrate sample was taken, using a glass jar 5.5 cm in diameter, and preserved in 10% formalin. From the intestine of each animal small samples of well mixed material were pipetted onto microscope slides. The sand grains were classified into groups by maximum dimension and counted. Particles less than 7 μm, which represent a minute fraction by volume (<1%), were not counted. Substrate samples were treated similarly and the sand grain size-frequency distributions were compared using a Mann-Whitney U-test (Sokal and Rohlf, 1981).

RESULTS

On the aboral surface, ciliary currents flow radially, centrifugally from the apex (Fig. 1). At the ambitus the direction of flow is downward, but with a continued centrifugal component. On the oral surface ciliary currents flow from anterior to posterior over the entire surface.

Cilia were found in two bands on opposite sides of all spines (Fig. 2, 3). The distance to which the bands extend up the spine is characteristic of each type. Fringe spines have an additional semi-circular band of cilia around the base, aborally (as noted previously by Mooi and Telford, 1982). Intercilium distances are approximately 1 μm. Although SEM micrographs (Fig. 3) indicate that cilia are 15–20 μm in length, light microscope measurements indicate that they are longer, up to 25 μm. This difference is possibly due to shrinkage during preparation. Echinoderm epidermal tissue is difficult to prepare for SEM, and even with critical point drying from acetone it shrinks. Because of the rigid underlying skeleton of calcite, the epidermis frequently tears with shrinkage, as the micrographs (Fig. 3) show.

Aboral club-shaped spines are oval in cross section at their distal ends. The orientation of the oval is not constant with respect to the longitudinal axis of the sand dollar (Fig. 1). Anteriorly, the major axis of the oval is perpendicular to an imaginary line passing through the apex and the spine. Posteriorly, the major axis is parallel to

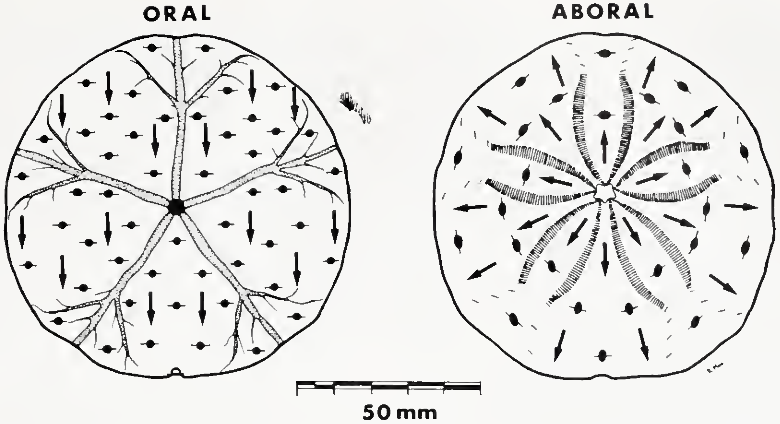

FIGURE 1. Ciliary currents and spine orientation on A) aboral and B) oral surface of *Echinarachnius parma*. Spines are shown as diagrammatic cross sections, circles (locomotory), and ovals (club shaped), with cilia at right angles to shaft. Orientation of the expanded tips of club-shaped spines is not constant but cilia on them are always arranged at right angles to lines radiating from the apex. Oral surface ciliary currents flow from anterior to posterior.

such a line. The orientation of the cilia on the aboral spines is independent of that of the spines themselves. On both club-shaped and miliary spines, it is always perpendicular to a line passing through the apex and the spine. Thus, these bands of cilia are arranged at right angles to the test radii. On the oral surface, cilia are oriented at right angles to the longitudinal axis of the test. In all observed instances currents

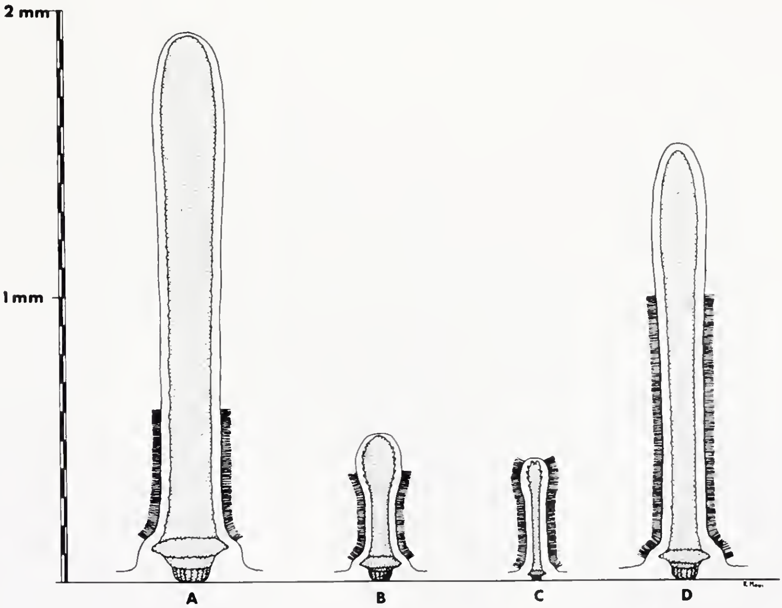

FIGURE 2. Distribution of cilia on major spine types: A) fringe, B) club-shaped, C) miliary, and D) locomotory. Fringe spines have an additional semi-circlet of cilia aborally around the base (not shown).

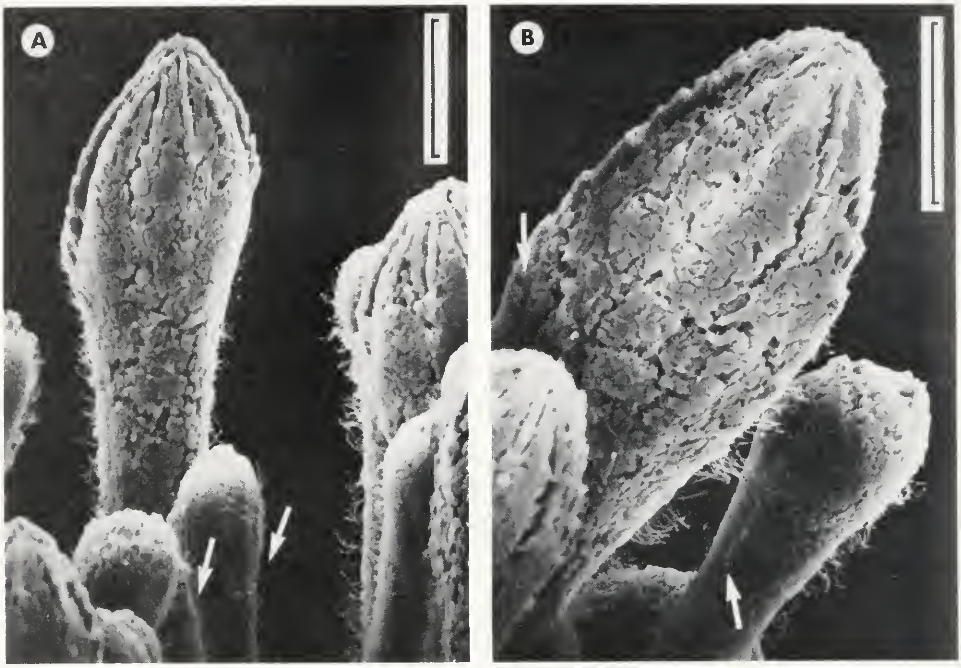

FIGURE 3. SEM micrographs of aboral miliary and club-shaped spines. Ciliary bands on some spines are clearly visible, arrows indicate those that are less conspicuous. Scale bar: 100 μm. A) Two diametrically opposed bands on club-shaped spine; B) more oblique view of another club-shaped spine and bands of cilia on a miliary spine.

flowed perpendicularly to the orientation of the ciliary bands, they were unidirectional and we never saw reversals of flow.

Between spines on the aboral surface, particles were carried to the ambitus by ciliary currents. The maximum size of particle (about 100 μm) that could be carried in this way is determined by the mean inter-spine distance (Mooi and Telford, 1982). In fact, even particles which were small enough to be transported between spines often bumped into them. This slowed their progress through the spine field and there was a negative correlation (-0.63; $P < 0.01$, $n = 32$) between particle size and net rate of transportation. For a 60 μm particle the rate was 0.08 mm s^{-1} and for a 10 μm particle 0.78 mm s^{-1}. The rate for small particles was probably maximal because they followed streamlines around spines while particles greater than 20 μm collided with them. At the ambitus small particles (10–20 μm) were swept downward around the edge of the test. Larger particles (>20 μm) were carried out of the aboral current, coming to rest near the middle or tips of the fringe spines before dropping to the substrate. An estimated 80% of particles (by number) reaching the ambitus via ciliary currents were lost in this manner. It was not possible to determine what proportion, if any, of the small particles successfully passed around the ambitus and were subsequently ingested. They could not be carried to the mouth by oral surface ciliary currents. Capture of particles from aboral ciliary currents by ambital or oral surface podia was not seen. Nearly all podium-particle contacts were with particles lying on the floor of the aquarium, only rarely with suspended particles.

Podia on the oral surface and at the ambitus picked up particles and pulled them towards the test where they were passed from podium to podium to the food grooves. In the food grooves they were similarly passed towards the mouth by podia. During this transportation, particles became progressively more aggregated by mucus, probably from the tips of the podia, which are known to have secretory cells (Mooi, 1983). Particles which had hitherto not stuck together, such as *Artemia* cysts, were aggregated with other particles after they had been carried along the food grooves. At the mouth, buccal podia pushed food clumps and diatom-covered sand grains into the opening. Food groove podia (60–80 μm diameter at the tip) were seen to extend to about the length of the oral surface locomotory spines, almost 1 mm. Ambital and aboral podia (100 μm tip diameter) can extend 5 mm or more from the test. Non-food groove podia on the oral surface (80–100 μm diameter) extend about 1.5–2 mm from the test, which allows them to reach the underlying sediment.

When concentrated diatoms or sand grains with numerous attached diatoms were offered as food, the rate of collection by podia and transport along the food grooves increased markedly. If concentrated diatoms were offered in only one ambulacrum, the podial activity in that ambulacrum increased while in the others it ceased. When this response occurred, the food grooves filled up with diatoms faster than they could be ingested. Sand grains covered with diatoms, bacteria, and debris were often ingested, as were clumps of filamentous diatoms and small adherent organisms. On two occasions nematodes in clumps of organic debris were masticated by pedicellariae and then passed to the mouth by podia. However, it appears that the contribution of pedicellariae to feeding is small and possibly accidental. Nothing comparable to the pedicellarial activity nor the spine-cone traps of *Dendraster* (Timko, 1976) was ever observed in *E. parma.*

Gut contents consisted of sand grains, diatoms, broken diatom frustrules, sponge spicules, crustacean fragments (parts of zoeae, ostracods, bits of exuviae, etc), and unidentifiable amorphous organic debris. The distribution of inorganic particle sizes in the gut was almost identical to that in the sediment. There were fewer large particles (>230 μm) but all other particles were represented in the same proportions. For small and intermediate sized particles, a Mann-Whitney U-test showed that there was no statistically significant difference between the proportions in the gut and sediment. The same non-parametric test showed that the proportion of the very largest particles (>230 μm) in the gut was significantly smaller than in the substrate ($P < 0.05$).

DISCUSSION

This study has demonstrated that in *Echinarachnius parma:* ciliary currents flow perpendicular to the orientation of cilia; aboral surface currents flow centrifugally to the ambitus but oral surface currents flow anterior to posterior; most particles carried by aboral ciliary currents are lost at the ambitus; podial collection of particles on the oral surface is a major source of food and the process is stimulated by the presence of diatoms; the inorganic particle size distribution in the gut is almost identical to the sediment except for the under-representation of particles greater than 230 μm. These observations indicate that the aboral sieving mechanism contributed little or nothing to feeding during these experiments.

The relationship between the orientation of cilia and the ciliary currents was described by Mooi and Telford (1982) but their description of centripetal ciliary flow on the oral surface was inaccurate. These currents are quite feeble and particles readily drop out of them. This makes it difficult to trace the flow by observing suspended material. Compared to the diameter of a sand dollar (about 50 mm), the field of view in a dissecting microscope at high magnification is quite small (about 4 mm) and this makes the significance of small differences in angle of flow difficult to perceive. Moreover, in the anterior ambulacra flow is towards the mouth or food grooves, thus appearing to support the centripetal current hypothesis when flow is not mapped over the entire surface. Most recently, Ghiold (1983) appears to have been misled in the same way when he reported centripetal oral surface currents in *E. parma.* There can be no doubt that many of the discrepancies in the literature and the sometimes warmly held differences of opinion, are due to the extreme difficulty of observing particle and current movements in the spine fields of sand dollars. Nonetheless, Parker and van Alstyne (1932) detected the anterior to posterior flow. In the present study the flow was first observed in small sand dollars, <10 mm diameter. It was subsequently confirmed by direct observation for all sizes. Precise mapping of the orientation of ciliary bands on oral surface spines provided a convincing morphological explanation of current flow. Mooi and Telford (1982) based their account on spines in the anterior region or too close to the food grooves and were misled by subtle changes of angles. The lunulate sand dollars might be quite different in this respect. Goodbody (1960) and Smith and Ghiold (1982) have reported centripetal oral ciliary currents in *Leodia sexiesperforata* and *M. quinquiesperforata* respectively. However, the observations made here that cilia are oriented with respect to the position of spines on the test, and not directly to the shapes of the spines, casts considerable doubt on currents inferred from spine orientation alone, as it has been for fossil species.

The conventional aboral rocking sieve mechanism initially postulated by Goodbody (1960) can be visualized in three steps: aboral collection and transport of particles to the ambitus, passage around the ambitus, and then transport across the oral surface to the mouth. Centrifugal aboral ciliary currents have been observed by all investigators. Hyman (1958) and Bell and Frey (1969) suggest that centripetal movement on the oral surface might rely more on podial activity than ciliary currents (except, of course, in *Arachnoides,* which lacks food groove podia). Our observations of the oral surface show that transport of food to the food grooves and mouth of *E. parma* is accomplished entirely by podia. Particles are passed from one podial tip to the next, "like a tiny bucket brigade," to borrow an expression from Mooi (1983). Thus, if the rocking sieve mechanism is to work, this leaves the crucial question of passage around the ambitus. No previous authors have explicitly stated that they observed it. Goodbody (1960) and Mooi and Telford (1982) clearly saw aboral and oral movement of particles but they viewed the two surfaces separately and only inferred that the flow of particles was continuous. There is no direct evidence in any report (Ghiold, 1979; Seilacher, 1979; Alexander and Ghiold, 1980; Lane and Lawrence, 1982; Smith and Ghiold, 1982) to confirm that transfer around the ambitus from aboral to oral surface really does occur. In this study we found that a very high proportion of particles was lost at the ambitus, especially those over 20 μm which tended to move out onto the fringe spines and fall off. Initial observations suggest, in fact, that the basal circlet of cilia around fringe spines might contribute a final thrust, ensuring the ejection of particles. Since the biggest particles are lost at the ambitus there would have to be a mechanism to catch the remaining particles (<20 μm) if this is, indeed, the feeding mechanism. Retrieval of such particles by podia near the ambitus was not observed. Podia were seen picking up particles from the substrate but not capturing them out of the ciliary flow. If a mechanism does exist for selection of such small particles, it should be reflected in the particle sizes of the gut contents.

Analysis of the gut contents indicates that for inorganic particles at least, there is no selection at all for small sizes (<100 μm) but there is some selection against large ones (>230 μm). The number of larger particles (230–500 μm) in the gut is about 60% that of the sediment. The hypothesis "there is a bias against large particles in the gut" can be distinguished from the alternative "there is a bias in favor of small particles in the gut." A real bias in favor of small particles would greatly inflate their representation because they make up such a large proportion by number of substrate particles. Conversely, a bias against large particles would change the proportion of small particles by number only slightly. The data support the hypothesis that there is a bias against large particles, not a selection for small ones. The gut content analysis, together with the failure to detect aboral to oral transfer of particles, clearly indicates that no aboral feeding mechanism was active in these animals. Certainly the frequent occurrence in the gut of particles (>100 μm) which could not be accommodated within the interspine spaces (25% by number, 45% by volume) indicates that the oral surface collection of particles by podia is a very important part of the feeding mechanism. Although this has not been detected previously, it should be noted here that Ghiold (1983) reported that particles, presumably suitable for ingestion, were collected by the ambital podia of *E. parma* and that the gut contained particles from 50 to 200 μm. It appears from the data of Lane and Lawrence (1982) that in *M. quinquiesperforata,* where interspine distances are similar to those of *E. parma* (Ghiold, 1983), as much as 30% by weight of the gut material is too large for the proposed sieve mechanism. Particles less than 100 μm could be collected directly by podia of *E. parma,* or, perhaps, trapped in mucus or adhering to larger particles. Jumars *et al.,* (1982) cite work by Valiella *et al.,* (1979) which describes deposit feeding mechanisms for dealing with cohesive sediments. This is probably important for *E. parma* also, because the sediments in which they live show some cohesive properties. For example, diatoms stick to sand grains, unidentifiable organic debris coats inorganic particles, and clumps of filamentous diatoms were found entangled with sand grains and debris. Many of these complex particles including diatoms, organic debris, and probably bacteria, were collected by the podia of *E. parma* and ingested. As in other deposit feeders, the rate of collection depends on the organic content, and in this particular case, the diatom concentration. In *Oreaster reticulatus* (L.), a sand-dwelling sea star, Schiebling (1980) reported that the rate of podial raking of the microphyte-rich layer was related to chlorophyll concentration and substrate particle size. Possibly oral surface feeding activity in *E. parma* is controlled in a similar way. It appears that the ambulacra can respond differentially because when only one ambulacrum is given diatoms, its activity level goes up while the other ambulacra may stop processing particles entirely.

Oral surface collection of substrate particles by *E. parma* is much like the mechanism described by Telford *et al.,* (1983) for *Echinocyamus pusillus* and does not differ radically from that of *D. excentricus* (Timko, 1976). Podia gather particles either by sucker action or by sticky mucus. Mucus is secreted from the tips of many podia (Mooi, 1983; Telford *et al.,* 1983) and may aid in forming a seal for the sucker. Podial collection and transport provides a viable alternative hypothesis to the aboral rocking sieve mechanism of feeding. It explains the enormous numbers of podia on the oral surface and is independent of surface ciliary currents, which might best be regarded as ventilatory and cleansing. The rapid response of podia to the presence of diatoms, the filling of the food grooves, and the visible ingestion of diatoms leaves no possible doubt that this is a major feeding mechanism in *Echinarachnius parma.*

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LUMINESCENCE CONTROL IN THE TUBE-WORM *CHAETOPTERUS VARIOPEDATUS:* ROLE OF NERVE CORD AND PHOTOGENIC GLAND

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ABSTRACT

Electrical and mechanical stimulation of the parapodial epidermis of *Chaetopterus variopedatus* evoked luminescence which was propagated only slightly to adjacent ipsilateral, but not to contralateral, parapods. In contrast, electrical stimulation of the highly modified aliform notopods led to propagation of luminescence through the entire body. Above a critical stimulus threshold, stimulation of the ventral nerve cord at any level evoked luminescence which was through-conducted. Only stimulation of the cerebral ganglia could bring about an orderly antero-posterior sequence of luminescence propagation. Discharges of nerve cord impulses invariably preceded the onset of spontaneous or electrically stimulated luminescence, and the propagation of both activities was interrupted by section of the nerve cord. Mechanical stimulation of parapods also evoked impulses at the corresponding level in the nerve cord. A large photogenic gland lying on the dorso-median surface of the 10–12th segments was refractory to electrical and mild mechanical stimulation, but responded by releasing large amounts of luminescent mucus after rupture of its epithelium. Mechanical agitation of the tube was quickly followed by the ejection of a cloud of luminescent mucus through one end, and readjustment of the worm's position to the other end of the tube. Epithelial luminescent activities are coordinated by the ventral nerve cord and luminescent discharges from the photogenic gland appear to be associated with defensive and tube cleaning activities.

INTRODUCTION

The bioluminescence of *Chaetopterus variopedatus* has puzzled naturalists for more than a century because little use could be made of light emitted by an animal always confined inside its tube, which is itself largely buried in sandy bottoms (Enders, 1909; Dahlgren, 1916; Harvey, 1952). Nicol (1962) postulated that the extracellular luminescent mucus produced by the worm's epithelium could serve to repel photonegative intruders, such as the small crustaceans often found inside the tubes (pers. obs.). These speculations have proved difficult to test experimentally or by field observations because of the secretive habits of this worm.

An alternative and more tractable approach is to make observations in the laboratory of behavioral activities accompanying light emission and to investigate the role of the nervous system in the coordination of luminescence. Previous investigators have focused on the effects of various stimuli on luminescence of "intact" worms (Nicol, 1952a, b, c, 1954) and of isolated notopods (Anctil, 1981), as well as the effects of putative transmitters on luminescent responses (Anctil, 1981). These studies established that: (1) single and repetitive shocks applied to the ventral nerve cord or

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to isolated parapods can elicit luminescence, (2) section of the nerve cord alters the normal propagation of the luminescent response, (3) the responses are subject to facilitation, summation, and fatigue, and (4) acetylcholine has excitatory and gamma aminobutyric acid (GABA) inhibitory effects on isolated parapods. These observations led to the conclusion that the luminescent epithelium is controlled by the nervous system and that antagonistic cholinergic and GABAergic pathways mediate this control at the periphery. Studies on the histology (Nicol, 1952a) and ultrastructure (Anctil, 1979) of the luminescent epithelia of *Chaetopterus* disclosed a subepidermal nerve plexus with some of its neurites making junctional contacts with musculo-epithelial cells. The latter are apparently responsible for the extrusion of the luminescent mucus through their squeezing action on the mucous cells (Anctil, 1979).

While the nervous system appears to be involved in the regulation of light emission in *Chaetopterus,* there is almost no information available on how neural coordination of luminescence is achieved. This information could provide clues as to possible uses the animal could make of its luminescence. The principal aim of this paper was to build on Nicol's (1952b, c) observations and elucidate the relationship between nerve cord activity and light emission in *Chaetopterus* using photometric and electrophysiological recording techniques. We also report an as yet undescribed photogenic gland whose triggering mechanism differs greatly from that of the regular luminescent epithelium.

Materials and Methods

Maintenance and handling of specimens

Natural parchment tubes containing specimens of *Chaetopterus variopedatus* were obtained from Pacific Bio-Marine Laboratories (Venice, California). They were maintained in an aquarium system containing aerated, filtered, and recirculated artificial sea water (Instant Ocean). Temperature (14–16°C), salinity (35.5 ppm), and pH (8.4) were checked regularly. The worms were kept in darkness except for short periods of handling.

Due to the extremely delicate texture of the specimens and the ease with which luminescence can be evoked accidentally, great care was taken in the handling of the worms. Since chemical anesthesia inhibited light emission, specimens were immobilized in cold sea water (0°C). Each tube was then opened and the resident worm immediately placed in a petri dish containing sea water precooled to 0°C. The specimen was pinned tightly through the distal ends of the parapods on the Sylgard-coated bottom of a petri dish prior either to photometric recordings or exposing the nerve cord for electrophysiological recordings. All experiments were carried out at 20–21°C.

Photometric and electrophysiological recordings

In addition to visual records of luminescence by the dark-adapted (20–25 min) observer, photometric recordings of light emission following mechanical and electrical stimulation also were made. For this purpose, a fiber light guide was connected to a photomultiplier tube (EMI 9601B) which was activated by a high voltage power supply (EMI Gencom 3000R). The signal was received by a Grass 7P amplifier and recorded on a Grass 79D polygraph. A manual shutter was operated between the light guide and the photomultiplier to permit distinguishing dark noise from spontaneous activities of the preparation.

For electrophysiology, extracellular recordings were made using glass suction electrodes with a diameter of 20–100 μm. A chlorided silver wire was introduced coaxially into the electrode tip which was polished over an alcohol burner. To avoid interference arising from the large quantities of mucus produced by the worm, good electrode contact was achieved by connecting the latter to a suction chamber; suction was controlled by connecting the chamber to a three-way valve and a 1-ml disposable syringe. The suction chamber was placed on a micromanipulator for precise positioning of the electrode tip viewed through a dissecting microscope.

Signals were fed to a Grass P511 preamplifier whose bandwidth was adjusted between 1 and 30 Hz. The amplified signals were received by a Tektronix 5113 oscilloscope for instantaneous visualization and by a Grass 79D polygraph for permanent records.

Stimulation

Electrical stimulation was applied through fine teflon-coated platinum electrodes which were connected to a Grass stimulation isolation unit and fed with square pulses from a Grass S44 stimulator.

Mechanical stimulation of limited epithelial areas of the parapods was delivered through a glass rod with a smooth, rounded end. The rod was clamped to a galvanometer (Grass 70SC D'Arsenval oscillograph). The application of D.C. current (10–50V) from a Grass stimulator to the galvanometer allowed control over the amplitude and duration of the displacement of the probe. The force delivered by this experimental set-up ranged between 0.015 (10V) and 0.038 dyne (50V) as estimated with a Grass FT.03 force transducer.

RESULTS

Electrical stimulation

Suprathreshold stimulation of any serial parapod by a single pulse led to a local luminescent response. As stimulus intensity was increased, luminescence spread to neighboring parapods, both anteriorly and posteriorly. First, ipsilateral parapods were recruited followed by contralateral parapods at higher stimulus intensities (Fig. 1).

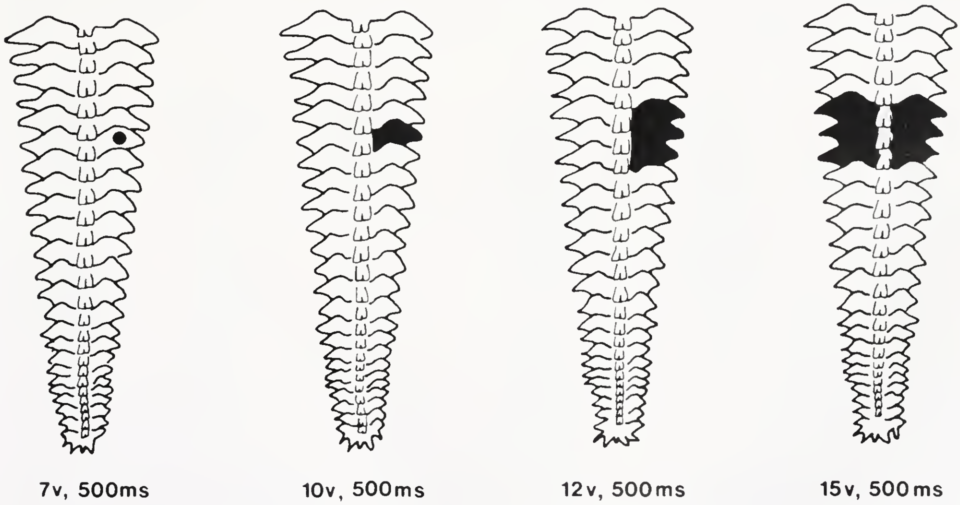

FIGURE 1. Schematic representation of recruitment of luminescent zones in posterior parapods in response to a single pulse of 500 ms of increasing intensity, applied to one parapod (see dot). Black regions represent sites of light emission.

The apparent intensity of the light emission of a parapod diminished as a function of its distance from the site of stimulation. Only a few segments could be recruited in this fashion, and luminescence was not propagated further either anteriorly or posteriorly in the 6 specimens tested.

A mild stimulation with square pulses (7V, 500 ms) on the surface of either aliform notopods of the 12th segment induced a weak, local luminescent response. With successive increases of stimulus intensity, luminescence became brighter and spread to the contralateral notopod, the middle segments, and eventually to all segments of the anterior and posterior regions, except for the photogenic gland of the 10–12th segments which remained unresponsive (see below). Thus, in contrast to other segments, the aliform notopods appeared to be preferentially accessible to the conduction pathway for luminescence propagation. The suspected substrate for this pathway, the ventral nerve cord, was then examined.

Stimulation of the nerve cord between segments 3 and 17 confirmed Nicol's (1952b) finding that the luminescent response spread in a discontinuous sequence, luminescence from posterior segments being recruited before that of middle or even anterior segments. Nevertheless, nerve cord stimulation consistently induced bilateral responses with a more rapid and widespread propagation of luminescence than notopodial stimulation. Reproducible propagation of luminescence in a more continuous antero-posterior sequence was achieved by electrical stimulation of the cerebral ganglion (see Martin and Anctil, 1984 for neuroanatomy). The same sequence of propagation was elicited by increasing the intensity (Fig. 2) or frequency of stimulation. Stimulation of the nerve cord at the level of the aliform notopods with single pulses or trains of pulses (Fig. 3) led to a response of low amplitude in these notopods and at the margin of the first fan in the 14th segment; then luminescence intensified and spread to other middle segments, and to the segments of the posterior and anterior regions following successive increases of stimulus intensity (Fig. 3).

Propagation of luminescent responses was more readily achieved in the antero-posterior than reverse direction. A stimulating pulse of 50 V, applied on the nerve cord of the last posterior segment, was necessary to elicit a response in the adjacent

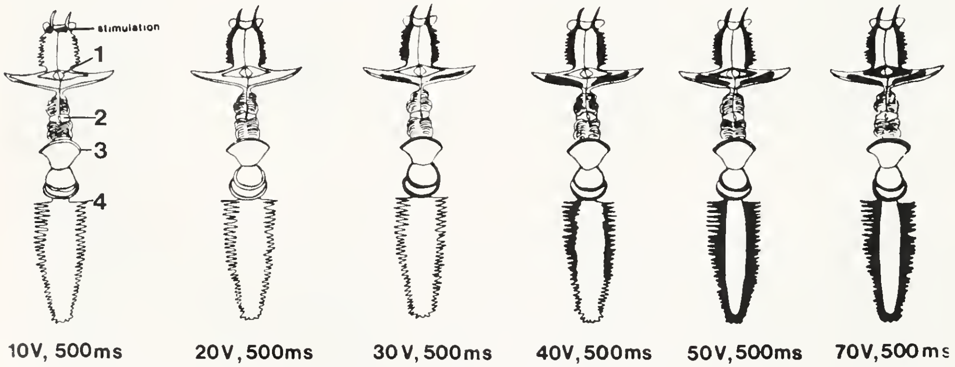

FIGURE 2. Schematic representation of recruitment of luminescent zones in response to a single stimulus pulse of increasing voltage, applied to the cerebral ganglion. Dot in extreme left specimen represents the site of stimulation and the black regions the sites of light emission. 1, Position of photogenic gland in 12th segment; 2, food cup in 13th segment; 3, first fan in 15th segment; 4, 18th segment where posterior segments begin. Note that photogenic gland in 12th segment failed to respond to a stimulus of 70V (extreme right). Data based on observations of six specimens.

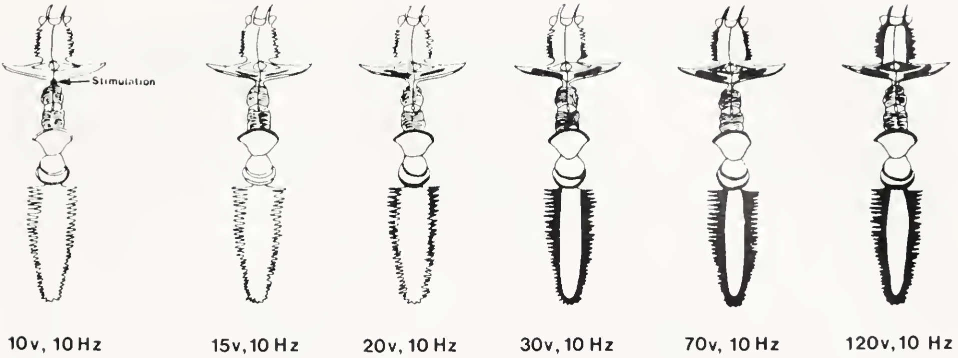

FIGURE 3. Schematic representation of recruitment of luminescent zones in response to pulse trains, lasting 2 s, of increasing voltage and applied to the nerve cord in the 12th segment. Symbols as in Figure 2. Data based on observations of six specimens.

segment. In contrast, a pulse of 10–20 V applied to more anterior segments was sufficient to elicit posterior spread in the same preparation.

Section of the nerve cord

The portion of the nerve cord associated with a single posterior segment was first isolated from the rest of the cord by double section. Electrical stimulation of this isolated portion elicited a light emission confined to both parapods of the associated segment in all specimens tested. Section of the nerve cord in the posterior region of the worm blocked the propagation of luminescence following electrical stimulation anteriorly (Fig. 4). Unilateral section of the widely separated left or right rami of the anterior nerve cord (see Martin and Anctil, 1984) had no effect on the propagation of bilateral luminescence, which still progressed posterior to the level of section. The conduction pathway for luminescence thus appeared to involve not only the two anterior nerve cords but also the numerous commissures interconnecting them.

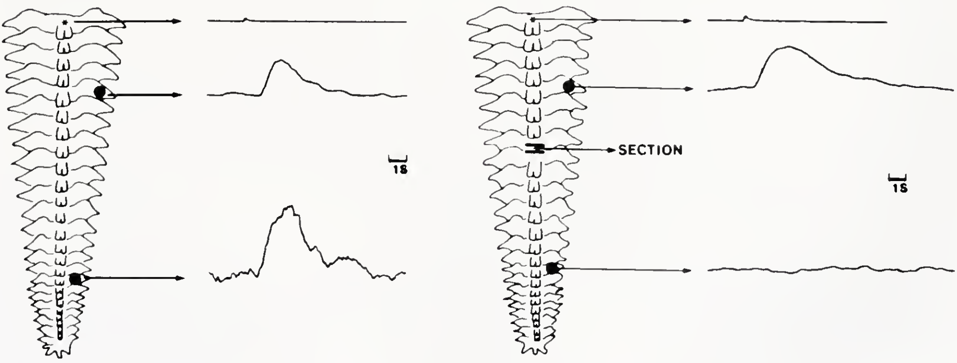

FIGURE 4. Effect of sectioning the nerve cord of the posterior region on propagation of luminescence. Left, intact nerve cord; right, after section of nerve cord. Upper trace, electrical stimulation (single pulse of 15 V, 500 ms); middle and lower traces, recordings of light emission. These observations were similarly obtained in all three specimens tested. Note failure of luminescence spread in the lower right recording.

Electrical activity in the nerve cord

Even in the non-luminescing worm the nerve cord continuously displayed arrhythmic signals, usually of very low amplitude. In contrast, light emission from any parapod was preceded by an increase in the electrical activity of the corresponding segmental ganglion in all 12 specimens investigated. This electrical activity was either spontaneous or electrically induced. Figure 5 illustrates two examples of simultaneous recordings of spontaneous impulse discharges and accompanying light emission.

These volleys varied in spike amplitude and frequency, and lasted between 0.5 and 2 s. The delay between initiation of the volley and onset of light emission ranged from 100 to 150 ms. Although the luminescent episodes varied in their rise and decay kinetics, no clear correlation between these and volley parameters could be made. Using two recording electrodes placed at varying distances from each other along the nerve cord, the conduction velocity of the volleys associated with luminescence was estimated to vary between 5 and 8 cm s^{-1}. The neural discharges associated with luminescence were conducted more readily in the antero-posterior than the reverse direction.

An additional volley often occurred near the onset or unfolding of the decaying phase of the luminescent response (Fig. 5a). Since the decaying phase reflects the chemical extinction of the extracellular luminescent reaction (Johnson, 1959) and/or the displacement of luminescent mucus away from the detection perimeter of the light guide, it is possible that this late discharge was associated with muscular activity resulting in mucus displacement.

Mechanical stimulation and the photogenic gland

Previous workers have stressed that the luminescent epithelium of *Chaetopterus* is highly sensitive to tactile stimulation or vibrations (see Nicol, 1952). This was confirmed by the difficulties in preparing the worms for experiments without inducing luminescence.

It was possible to produce controlled tactile stimulation on the epithelium using a galvanometer-controlled probe. Such a stimulus, applied to parapods, invariably induced a local luminescent response which failed to propagate to adjacent parapods despite the fact that it elicited a short impulse discharge in the segmental ganglion corresponding to the stimulated parapod (Fig. 6). This discharge, whose amplitude

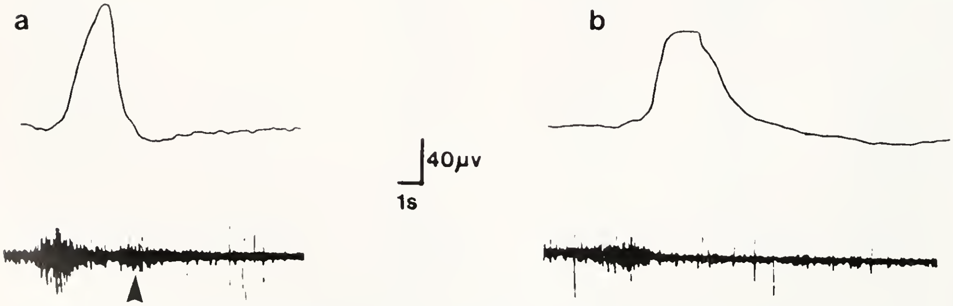

FIGURE 5. Recordings illustrating the relationship between spontaneous electrical activity in the nerve cord (lower trace) and spontaneous luminescent events (upper trace). In A the impulse discharge lasted 1.2 s and accompanying luminescence, 4 s; in B these parameters were of longer duration (2.2 s and 7.5 s, respectively). Note the after-discharge (arrowhead) in the nerve cord in A.

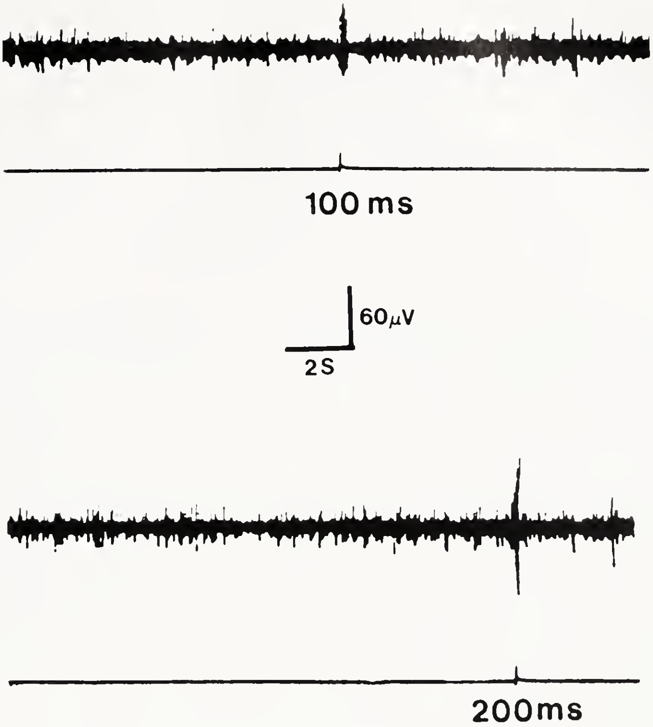

FIGURE 6. Recordings of electrical activity in the nerve cord (upper trace) in response to mechanical stimulation (.015 dyne) of the parapodial epidermis from the corresponding posterior segment (lower trace). Note the increment of impulse amplitude as a function of stimulus duration. Similar responses were obtained in all three specimens tested.

was dependent on stimulus strength or duration, was of much shorter duration than those associated with propagated luminescent responses (Fig. 5).

The photogenic gland appears as a swollen mass, 2–3 mm in diameter, and yellowish in live, sexually mature animals. It extends dorso-medially from the 10th segment to the intersection of the lateral and longitudinal ciliated grooves in the 12th segment (Fig. 2). Observations on serial histological sections from formalin-fixed, paraffin-embedded glands, stained with Mallory's triple stain, revealed that the gland consists of local enlargements of the glandular epithelium filled with mucous cells in a layer 700–800 µm deep at the expense of other cell types such as supportive and myoepithelial cells (Anctil, 1979). The photogenic gland, which was unresponsive to electrical stimulation either of the notopodial surface or of the nerve cord (Figs. 2, 3), consistently responded to direct mechanical handling by the release of a large quantity of brightly luminescent mucus in the water. The type of stimulation necessary to produce such a response (pinching or application of pressure on the gland) caused a rupture of the overlying epithelium of these glands, as visualized through a dissecting microscope. Thus epithelial rupture was an apparent prerequisite for expulsion of the mucus.

In order to assess the significance of mechanical disturbances in the context of tube-dwelling, eight tubes with their resident worm still inside were subjected to mechanical agitation in an aquarium. Following vigorous shaking of one extremity of the tube with the fingers, a copious amount of luminescent mucus was almost instantaneously expelled through the agitated end in all trials (Fig. 7-1). The intense and bluish luminous cloud thus produced in the surrounding water was visible to the dark-adapted eye for 2–3 min. A quick examination of the inside of the tube revealed that this forceful release was immediately accompanied by a complete reversal of the worm's orientation in the tube (Fig. 7-2), the worm now positioned in a contracted state at the other end of the tube.

DISCUSSION

This study conclusively establishes that except for the photogenic gland of segments 10–12 the nerve cord has an essential role in the excitation and propagation of the epithelial luminescent response of *Chaetopterus.* The cerebral ganglia, which are poorly developed in *Chaetopterus* (Martin and Anctil, 1984), may play a role in the coordination of luminescence propagation but are not necessary for the propagation itself. This is shown by the orderly spatial sequence of luminescence propagation following stimulation of a cerebral ganglion and the experiments on electrical stimulation of intact or sectioned nerve cord.

The luminescent responses to electrical stimulation of parapods and notopods are probably mediated by the nervous system for the following reasons: (1) luminescence spreads, albeit to a limited extent, as a function of the intensity of stimulation, (2) electrical stimulation of the aliform notopods, which are characterized by a much greater concentration of neurites than the parapods of the other segments (Anctil, 1979; Martin and Anctil, 1984), is the only means, short of direct nerve cord stimulation, by which to achieve through-conduction of luminescence excitation, and (3) a well-developed subepidermal nerve plexus is present throughout the body wall (Anctil, 1979; Martin and Anctil, 1984) and may act as the medium for decremental conduction of excitation at the periphery. It is unlikely, however, that intersegmental propagation of luminescence is mediated directly through the plexus since the latter does not appear to function as a true nerve net, at least in oligochaetes and polychaetes (Prosser, 1935, 1950; Mill, 1978). Furthermore, sectioning the nerve cord while leaving

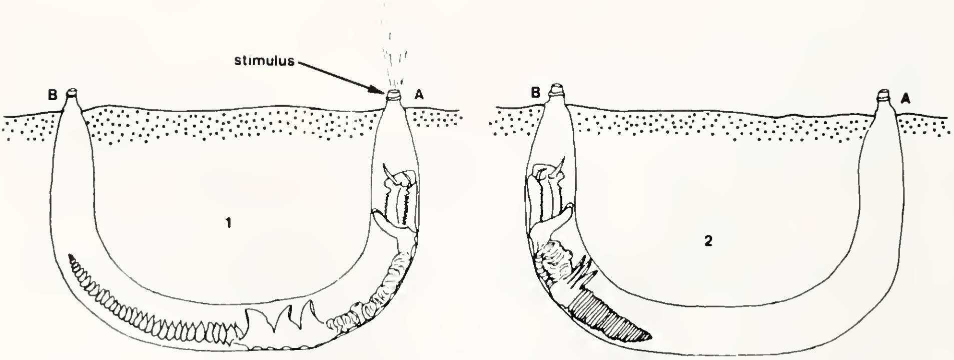

FIGURE 7. Schematic representation illustrating the relationship between the expulsion of luminescent mucus (1A) and the reversal of the worm's position at the other end of the tube (2B) in response to mechanical agitation of the tube. See text for further explanation.

the plexus intact is sufficient to block intersegmental propagation of stimulated luminescence.

Stimulation experiments on parapods suggest that the limited propagation of luminescence thus produced is dependent on a relatively small number of neurites recruited for conduction and on synaptic barriers that the nerve cord must overcome to transmit the excitation across the connectives and commissures linking the segments and the two cords, respectively. This is substantiated by the local luminescent response and accompanying nerve cord activity elicited by the mild mechanical stimulation of a small patch of parapodial epidermis. This presumably small sensory field would command a limited number of afferents to the nerve cord, probably insufficient to overcome the threshold level set by the synaptic layout of the local ganglionic neuropile.

In contrast, propagation of luminescence through the entire body is readily achieved by nerve cord stimulation. We have identified impulse discharges which travel along the ventral nerve cord and are associated with luminescent episodes, either spontaneous or electrically stimulated. These temporally correlated activities provide additional support for the necessary role of nerve cord function in activating the luminescent epithelium. The high spiking frequency as well as the small and variable amplitude of spikes in these volleys probably reflect the activity of multiple neurites of small caliber. This view is consistent with measurements of axon diameters of 2 μm or less in the nerve cord of *Chaetopterus* (Martin and Anctil, 1984).

Two features of the nerve cord-mediated luminescence conduction deserve mention: the conduction velocity and the polarization of impulse conduction. The slow speeds of conduction (5–8 cm s^{-1}) are within the range reported in the nerve cord of other annelids where giant fibers are not involved and multiple synaptic interactions between segments occur (Bullock and Horridge, 1965). No giant fiber has been detected in *Chaetopterus* (Nicol, 1948; Martin and Anctil, 1984), and the great majority of the nerve fibers in the nerve cord were found to be less than 1 μm in diameter (Martin and Anctil, 1984). Bullock and Horridge (1965) reviewed evidence of a "preference for posterior propagation" in the nerve cord of polychaetes which we substantiate in the case of *Chaetopterus*. The functional morphology on which this behavior is based is uncertain, although the presence of a large number of polarized and of relatively few non-polarized (symmetrical) synapses in segmental nerves (Anctil, 1979) as well as in the nerve cord (Martin and Anctil, 1984) could be a contributing factor.

Discrete mechanical stimuli readily induced luminescent responses in *Chaetopterus*. These local responses might be elicited through a reflex pathway involving sensory cells, such as the ciliated cells in the epidermis of *Chaetopterus* (Anctil, 1979), whose excitation would be transmitted to the subepidermal nerve plexus and finally to the myoepithelial cells mediating mucus release. The nerve cord activity recorded following stimulation of the same segment was probably induced by the subepidermal plexus. Therefore it is likely that large patches of sensory epidermis must be simultaneously activated to initiate propagated nerve cord impulses and consequent spread of luminescent responses such as is witnessed by vigorously handling the worm.

The refractoriness of the photogenic gland to electrical or mild mechanical stimulation, which has not been reported by previous investigators, is of particular interest in view of its large store of luminescent mucus and impressive luminescent display. What could be the biological significance of a structure that must be damaged in order to elicit a luminescent discharge? Brown and Rosen (1978) described a tube cleaning behavior in *Chaetopterus* involving a reversal of the orientation of its body inside the tube. This reversal is accomplished by raising and folding the anterior region of the body in the posterior direction, and this is accompanied by a torsion of the body. This is likely to cause a marked compression of the photogenic gland, leading possibly to the rupture of its epithelium and the propulsion of a large amount of luminescent mucus. The change of position followed by vigorous movements of the fans of the mid-region of the body (Brown and Rosen, 1978) cause a reversal of the direction of the water flow in the tube and, consequently, the forceful expulsion of luminescent mucus as well as undesirable detritus material or intruders. The propulsive force is enhanced by the locomotory activity of the worm toward the opposite end of its tube which is in the same direction as that of the water pumping activity.

Thus, according to our hypothesis, the photogenic gland is not under direct nervous control but becomes involved directly as a result of mechanical stress produced by motor activities associated with the tube-cleaning behavior or mechanical agitation of the tube. Indeed, our observations show that vigorous mechanical agitation of the tube not only induces a forceful ejection of luminescent mucus, but also causes the reversal of position and locomotory activity previously reported by Brown and Rosen (1978). It is also possible that the luminescent mucus from the regular luminescent epithelium of the parapods is released following multiple disturbances comparable to those causing the ejection of mucus from the photogenic glands. It should be emphasized however that the amount of mucus the luminescent epithelium can mobilize in such a short time is insufficient to account for the impressive cloud of luminescence witnessed under these conditions.

These observations suggest a possible role for bioluminescence in *Chaetopterus*. The worm appears to react similarly to invasion of its tube by foreign materials and outside attacks on the tube. The reaction involves a defensive retreat of the worm to one end of the tube and the expulsion of a bright cloud of luminescent mucus at the opposite end. The dispersal of that cloud in the water would then confuse the potential attacker as to the exact location of its prey. There are still unresolved problems with this model, namely the relative contributions of the photogenic gland and neurally controlled luminescent epithelium to the expelled luminous cloud, and the role of the ventral nerve cord in the integration of the sensoro-motor activities associated with this behavior.

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THE EFFECTS OF CALCIUM ON SEROTONIN-STIMULATED ADENYLATE CYCLASE IN FRESHWATER MUSSELS

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ABSTRACT

When serotonin (5-HT) is added to a pondwater bath (final concentration, 10^{-4} M/l) containing freshwater mussels, sodium influx is rapidly stimulated, as are behavioral responses. Serotonin also stimulates adenylate cyclase (AC) in gill homogenate pellets from two different freshwater bivalve families, suggesting that a serotonin-cAMP system is associated with control of Na transport and that this system may be common in many ion regulating mussel species. Adenylate cyclase activity was detected in several tissues of *Ligumia subrostrata;* the highest activity being observed in the foot. Endogenous calcium depressed the AC activity measured under basal, 5-HT, and dopamine stimulated conditions; the reduced enzyme activity was most pronounced in the monoamine stimulated preparations. AC activity of the crude homogenate pellet was significantly lower than a purified pellet formed by an additional 100 × *g* centrifugation prior to a 3000 × *g* centrifugation. Exogenous calcium (2.5 mM/l) inhibited monoamine stimulated AC activity about 50%. Calcium concretions exist in gill tissue and may influence the observed AC activity by increasing the protein measure or increasing the calcium concentration. Prostaglandin E_2 had no effect on basal or 5-HT stimulated AC activities in the purified pellet. Although phosphodiesterase and non-specific phosphatase activities were high in the supernatant, their activities in the homogenate pellet were low and had little effect on the AC activity measurements.

INTRODUCTION

Freshwater mussels maintain blood ion concentrations (Na, Cl, Ca, and HCO_3) above the level in pondwater (Dietz, 1979). Sodium and chloride transport are independent of each other in freshwater mussels (Scheide and Dietz, 1982); the gill is the primary site of Na accumulation (Dietz and Findley, 1980; Dietz and Graves, 1982). Although the mechanisms regulating each of these ions have not been elucidated, sodium ion regulation appears to be one function of a serotonin (5-HT)-coupled adenylate cyclase system in freshwater bivalves (Dietz *et al.*, 1982; Scheide and Dietz, 1983). Thus, a 5-HT stimulated adenylate cyclase (AC) is present in mussel gill tissue; a 5-HT dose-related effect on sodium influx has been observed; and gill cAMP levels are directly related to sodium transport rates (Dietz *et al.*, 1982; Scheide and Dietz, 1983).

The AC activity of *Ligumia subrostrata* gill tissue varies from mussel to mussel (Scheide and Dietz, 1983). Adenylate cyclase is a calcium sensitive enzyme, but the sensitivity is biphasic with 1–100 μM/l calcium activating AC whereas 0.1 to 1.0 mM/l inhibits the enzyme (Hynie and Sharp, 1971; Bockaert *et al.*, 1972; Brostrom

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et al., 1977). Since calcium concretions occur in the gills (Silverman *et al.,* 1983), we examined the effects of Ca on AC activity in mussel gill homogenate as a possible source of AC variability.

In this report, we present information on sodium transport stimulation by serotonin in several species of freshwater mussels and relate this to the 5-HT stimulated adenylate cyclase. We also present data on the 5-HT stimulated AC in several tissues in *Ligumia subrostrata.*

MATERIALS AND METHODS

All organic chemicals were from Sigma Chemical Co. The ^{22}Na used in flux determinations was from New England Nuclear and the ^{3}H-cAMP (26 Ci/m*M*) for the binding protein assay was from Amersham.

Representative members of the Unionidae (*Anodonta grandis, Carunculina texasensis, Ligumia subrostrata*) and Corbiculidae (*Corbicula fluminea*) were collected locally and stored in aerated artificial pond water (0.5 m*M*/l NaCl, 0.4 m*M*/l $CaCl_2$, 0.2 m*M*/l $NaHCO_3$, and 0.05 m*M*/l KCl). These bivalves were acclimated to laboratory conditions for at least five days prior to experimentation. Males were used when possible to avoid interference by the brooding of glochidia.

Blood samples were taken by cardiac puncture and from the ventral edge of the foot. Handling mussels when the foot is extended results in a stream of blood from a "hemal pore" in the foot (see Greenaway, 1970). This blood was collected by directing the fluid into a centrifuge tube. Blood collected by cardiac puncture or from the foot was centrifuged for 1 min at 8000 × *g* and analyzed by flame photometry (sodium), electrometric titration (chloride), and atomic absorption spectrophotometry (calcium).

Sodium fluxes were analyzed to show the immediate effect of serotonin (5-HT) on sodium transport. Bivalves were rinsed for about 1 h in distilled H_2O (DW) and then placed in individual containers of pondwater containing ^{22}Na (Graves and Dietz, 1982). Once the animals had opened and had commenced siphoning, flux determinations were started with the removal of a bath sample. Basal flux periods averaged about two hours; then serotonin (final bath concentration of 10^{-4} *M*/l) was added to the pondwater medium and mixed gently to minimize the disturbance to the animals. Within 15 min the bivalves had opened again and were siphoning and the serotonin flux measurement was initiated by withdrawing a bath sample. The average flux period during 5-HT stimulation was one hour, then the final pondwater bath sample was removed.

Sodium net flux was determined by monitoring the change in the sodium concentration of the bath for several hours. The bivalve soft tissue was removed, dried at 90°C, and weighed. The net flux was expressed as μeq $(\text{g dry tissue} \cdot \text{h})^{-1}$. Sodium influx was determined by monitoring the disappearance of ^{22}Na from the pondwater bath; the efflux was calculated by: $J_{out} = J_{in} - J_{net}$.

The adenylate cyclase assay is detailed in Scheide and Dietz (1983). Briefly, tissue was homogenized in 5 ml 50 m*M*/l tris-SO_4, pH = 7.6 buffer with a Tissumizer (Tekmar) and centrifuged at 3000 × *g* for 20 min at 4°C. The surface of the crude homogenate pellet was rinsed with 2.0 ml tris-SO_4 buffer and then resuspended in 5 ml buffer. A purified pellet was produced as follows. The crude homogenate was first centrifuged at 100 × *g* for 20 min (4°C). The supernatant was decanted and recentrifuged 20 min at 3000 × *g*. The resulting purified pellet was rinsed in 2 ml of buffer and resuspended in 5 ml buffer. This procedure removed the large cells, cellular fragments, and the calcium concretions (see Silverman *et al.,* 1983).

Adenylate cyclase activity was determined by incubating the resuspended pellet, at room temperature, in a mixture of 50 m*M* tris-SO_4, pH = 7.6, 10 m*M* $MgSO_4$, 2 m*M* theophylline, 0.5 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5 m*M* adenosine 5′ triphosphate, 0.1 m*M* guanosine 5′ triphosphate, 2.5 m*M* creatine phosphate, 2.5 units per test tube creatine phosphokinase (25°C), and other additions as noted in the Results (neurotransmitters and $CaCl_2$). The reaction was initiated by adding the resuspended pellet to give a total incubation volume of 200 μl; it was terminated after 5 min by adding 0.8 ml boiling water and boiling the reaction tube for 3–5 min. Cyclic AMP formed during the assay was quantified with a cAMP binding protein isolated from human red cells (Scheide and Dietz, 1983). Adenylate cyclase activity was expressed as pmol cAMP (mg protein · 5 min)$^{-1}$. Protein values were determined by the Lowry method (Lowry *et al.*, 1951).

The AC activity was determined in homogenates of gills from the 4 species studied. Homogenates were resuspended to give between 0.12 and 0.17 g original wet gill tissue weight per ml of resuspended pellet. Dilution of various *Ligumia subrostrata* tissue crude homogenate pellets averaged 0.13 to 0.21 g original wet tissue weight per ml of resuspended pellet for gill, mantle, foot, heart, and muscle.

Endogenous calcium values were determined by centrifuging the resuspended homogenate pellet at 8000 × *g* for 1 min and diluting a known volume of supernatant in 1% LaO_3–5% HCl. This procedure separated the particulate calcium concretions in the homogenate from the "soluble" supernatant calcium. Calcium was measured with a Perkin-Elmer 303 atomic absorption spectrophotometer.

Cyclic AMP degrading enzymes were assayed by incubating 300 pmol cAMP in a reaction tube with a final concentration of 50 m*M*/l tris-NO_3, pH = 7.6, 10 m*M*/l $Ca(NO_3)_2$. Gill tissue from *Ligumia subrostrata* was homogenized in 3–5 ml 50 tris-NO_3, pH = 7.6 buffer. Tissue fractions were obtained after a 3000 × *g* centrifugation for 20 min (4°C). The pellet was resuspended in tris-NO_3 buffer, 1 ml per 0.21 g wet tissue weight. The supernatant was from a homogenate formed by 0.14 g wet tissue weight per ml of homogenate buffer. The phosphatase assay was initiated by the introduction of the supernatant fraction (final volume, 0.2 ml) and terminated by the addition of 0.8 ml boiling water followed by boiling the reaction tube for 3–5 min. The cAMP remaining was determined by use of the cAMP binding protein (Scheide and Dietz, 1983). A zero activity control was prepared by boiling (3–5 min) the supernatant fraction (diluted with 0.8 ml DW), then adding tris-NO_3/$Ca(NO_3)_2$ buffer and cAMP. Protein determinations were by the method of Lowry *et al.* (1951). Enzymatic activities were determined by subtracting the cAMP concentration measured in each reaction tube from that of the zero activity tube and were expressed as pmol cAMP degraded (mg protein · 5 min)$^{-1}$. All assays were done in triplicate. Phosphodiesterase activity was determined by subtracting the cAMP present per reaction tube containing 4.0 m*M* theophylline from the cAMP remaining in a non-theophylline containing reaction tube. The tube with theophylline represented non-specific phosphatase (referred to as phosphatase) activity. Preliminary results indicated this reaction to be linear for the first 10 min.

Data are expressed as mean ± standard error. Statistical analyses utilized were the two-tailed Student's *t*-test and least-squares linear regression.

RESULTS

Sodium transport in the four species was stimulated by 5-HT (10^{-4} *M*/l in pondwater) (Table I). All four species exhibited the same "serotonin response" observed previously when 5-HT was injected into the foot of the mussel (Dietz *et al.*, 1982).

TABLE I

Control and serotonin (10^{-4}M/l) stimulated sodium transport in four representative mussels immersed in pondwater

		SODIUM FLUX μeq (g dry tissue · h)$^{-1}$					
		Control			5-HT Stimulated		
Species	n	J_{net}	J_{in}	J_{out}	J_{net}	J_{in}	J_{out}
Anodonta grandis	6	−0.27 ± 0.35^A	0.45 ± 0.29	0.72 ± 0.63	−1.63 ± 0.63	2.29 ± 0.66^B	3.92 ± 0.33^C
Carunculina texasensis	6	−0.41 ± 0.10	0.63 ± 0.20	1.04 ± 0.19	1.55 ± 0.32^C	2.01 ± 0.32^{CE}	0.46 ± 0.16^B
Corbicula fluminea	5	−1.89 ± 0.93	4.62 ± 0.85	6.51 ± 1.52	8.18 ± 0.94^D	10.59 ± 1.28^{CE}	2.41 ± 0.65^B
Ligumia subrostrata	5	0.67 ± 0.83	1.18 ± 0.65	0.51 ± 0.25	5.00 ± 0.50^D	5.60 ± 0.77^{CE}	0.60 ± 0.32

[A] Values represent the mean ± the standard error of the mean.
Values significantly different from controls, B: $P < 0.05$
C: $P < 0.01$
D: $P < 0.001$.
Values of J_{in} and J_{out} significantly different E: $P < 0.01$.

The control flux of each individual was determined immediately before the addition of 5-HT.

The reaction to serotonin treatment includes an increase in siphoning, pronounced valve gaping movements, an extension of the foot, and increased locomotor activity. The 5-HT stimulated sodium influx in each species was 2.5 to 5 fold over the basal sodium influx. In *Carunculina texasensis, Corbicula fluminea,* and *Ligumia subrostrata* 5-HT stimulated the influx significantly above the efflux resulting in a significant ($P < 0.01$) net movement of sodium into the mussel. However, in *Anodonta grandis,* the sodium efflux was significantly increased ($P < 0.01$), in addition to the stimulation of the influx, resulting in a net loss of sodium. The loss of sodium by *A. grandis* could have been due to a serotonin effect on the excretory component, a loss of fluid from the foot during valve gaping and locomotor movements which occurred throughout the flux interval, or from the disturbance associated with bath sample withdrawal.

Analysis of the foot blood (see Materials and Methods) indicated that although its ion concentrations were approximately 47% and 64% of those in the blood of *A. grandis* and *L. subrostrata,* the ratios of the ions in the two fluids were similar (Table II). The low concentrations in the expelled fluid and the high sample variability probably resulted from dilution by pondwater present in the mantle cavity and gill chamber. On two occasions when a fluid stream was directed horizontally and collected in a beaker held several cm from the animal, the Na concentration was higher than the fluid dripping directly from the ventral edge of the foot (18.8 ± 0.1 meq Na/l for *A. grandis,* n = 2). The Na, Cl, and Ca concentrations in the fluid collected from the foot were between 5 to 17 times greater than those in the pondwater.

Adenylate cyclase activity was measured in the resuspended crude homogenate pellet of gill tissue from the four mussel species (Fig. 1). Serotonin increased the AC activity ($P < 0.01$), by 3–5 times the basal level in *A. grandis, C. fluminea,* and *L. subrostrata.* In *C. texasensis* the increase in AC activity by 5-HT was greater than 30 times the basal level; but the enzyme activities produced by 5-HT stimulation were similar in all of the species. Basal AC activity levels were significantly lower ($P < 0.01$) in *C. texasensis* when compared to basal values of *A. grandis, C. fluminea,* and *L. subrostrata.* The trends in AC activity, normalized to Lowry protein concentration, were essentially unchanged when AC activity was expressed per gram wet tissue. *Anodonta grandis, C. fluminea,* and *L. subrostrata* had similar gill homogenate pellet AC basal and 5-HT stimulated activities: *A. grandis,* 1158 ± 289 *versus* 3658 ± 925 (n = 6); *C. fluminea,* 1147 ± 319 *versus* 4384 ± 1048 (n = 4); and *L. subrostrata,* 1499 ± 202 *versus* 3566 ± 439 (n = 6) pmol cAMP (g wet tissue · 5 min)$^{-1}$. *Carunculina*

TABLE II

Blood and foot ventral edge fluid collected from Anodonta grandis *and* Ligumia subrostrata *after a 1.5 h exposure to* 10^{-4} M/l *5-HT pondwater*

	ION CONCENTRATION mM/l							
	Blood				Foot Fluid			
Species	n	Na	Cl	Ca	n	Na	Cl	Ca
Anodonta grandis	10	19.3 ± 0.7[A]	17.0 ± 0.6	3.7 ± 0.2	4	12.2 ± 4.0	10.2 ± 3.6	2.6 ± 0.7
Ligumia subrostrata	11	20.1 ± 0.3	13.7 ± 0.4	3.5 ± 0.1	4	9.2 ± 1.9	6.2 ± 1.6	1.7 ± 0.3

[A] Values represent the mean ± the standard error of the mean.

Pond water values for the ions in this table were 0.7, 1.2, and 0.3 mM/l for Na, Cl, and Ca, respectively.

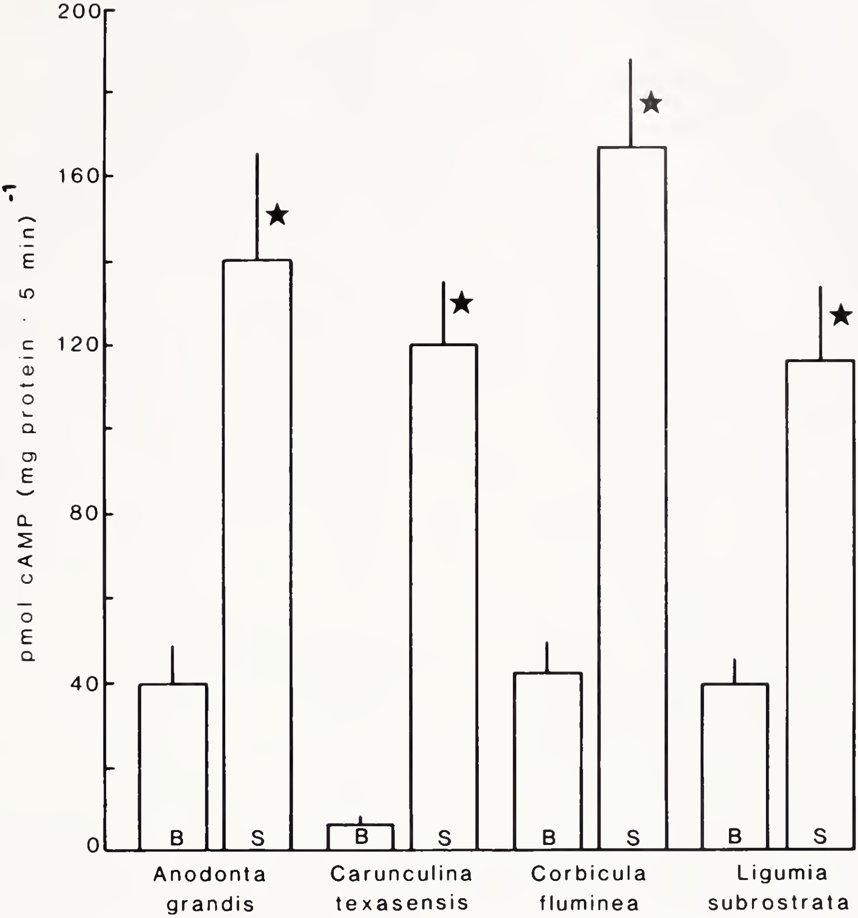

FIGURE 1. Basal (B) and 5-HT (S) (60 $\mu M/l$) stimulated AC activity in four species of freshwater mussels. Each corresponding bar represents the mean of five *Anodonta grandis*, four *Carunculina texasensis*, four *Corbicula fluminea*, and six *Ligumia subrostrata* gill tissue crude homogenate pellets assayed in triplicate. The vertical lines represent the standard errors of the mean. ★ = significantly different from corresponding basal values, $P < 0.01$.

texasensis exhibited 75.9 ± 46.5 *versus* 2828 ± 391 pmol cAMP (g wet tissue · 5 min)$^{-1}$ for basal and 5-HT AC activities (n = 4).

Adenylate cyclase was present in all *L. subrostrata* tissues studied (Fig. 2). Serotonin is a major neurotransmitter substance in bivalves and it significantly stimulated AC activity in each tissue. Basal AC activities observed in the gill, mantle, heart, and posterior adductor muscle were similar as were the 5-HT stimulated activities, displaying a 2.2 to 4.6 fold increase over basal AC activity. However, the foot muscle basal and the 5-HT stimulated AC activities were significantly higher ($P < 0.02$) than those in the other tissues. The same trends were evident whether AC activities were expressed in terms of gram wet tissue weight or normalized to Lowry protein content.

Adenylate cyclase activity values are quite variable between animals (this study, Scheide and Deitz, 1983). To better understand this variability, the relationship between AC activity and the endogenous calcium content was studied (Fig. 3). Observed AC

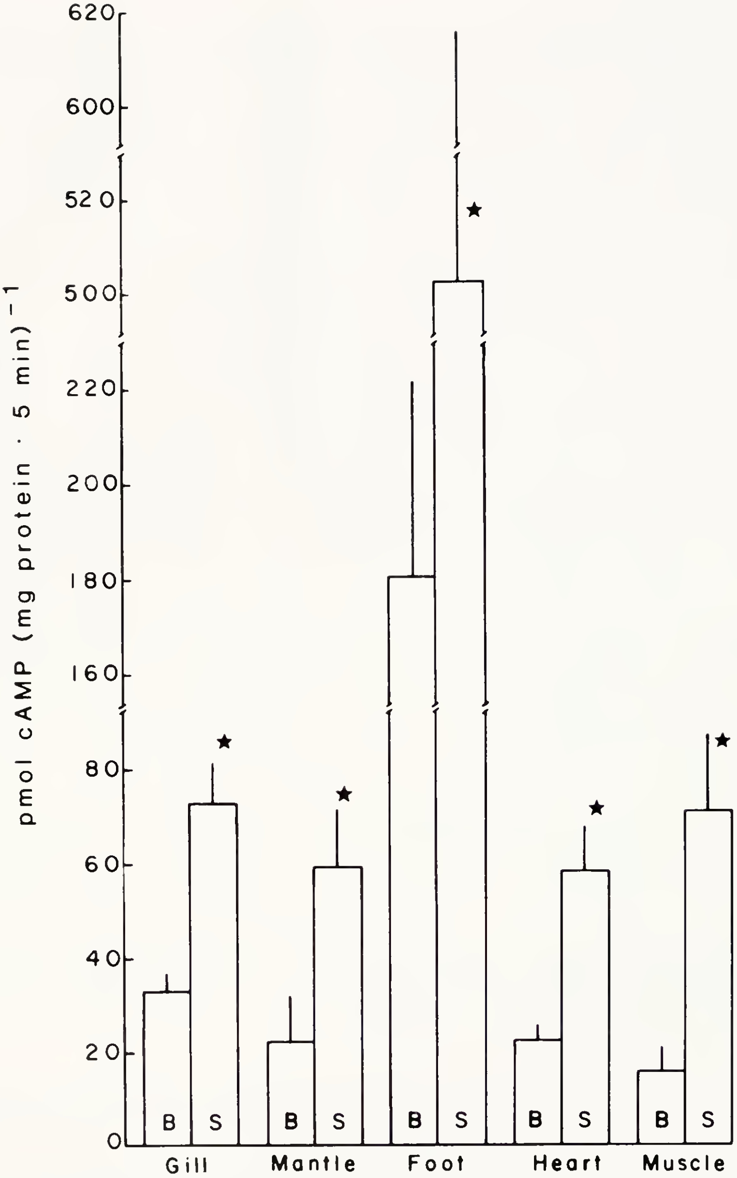

FIGURE 2. Basal and 5-HT (60 μM/l) stimulated AC activity in the various tissues of *Ligumia subrostrata*. The bars represent the mean of seven gills and three each of mantle, foot, heart, and posterior adductor muscle, each assayed in duplicate using crude homogenate. Vertical lines represent the mean standard error. ★ = significantly different from corresponding basal values, $P < 0.05$.

activity was inversely dependent on the endogenous calcium in the homogenate pellet. Basal ($r = 0.77$, $P < 0.001$), serotonin ($r = 0.88$, $P < 0.001$) and dopamine ($r = 0.79$, $P < 0.001$) stimulated AC activities were inversely related to endogenous calcium with the calcium appearing to have a greater effect on the monoamine (5-HT and dopamine) stimulated activity than the basal activity. Basal AC activity *versus* en-

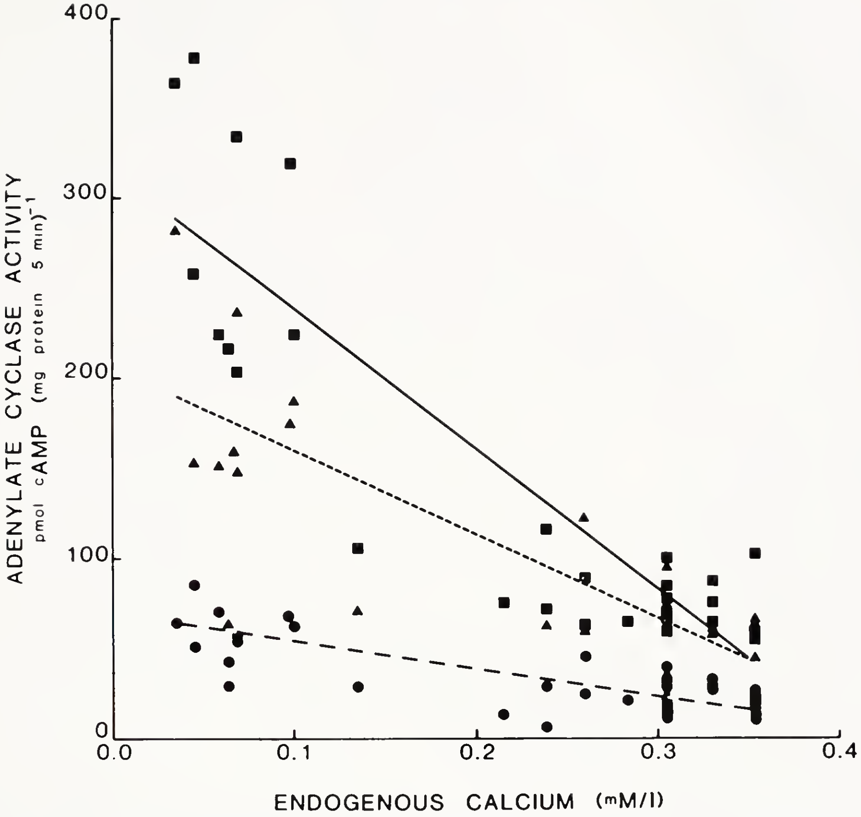

FIGURE 3. Gill adenylate cyclase activity varies inversely as a function of endogenous calcium in *Ligumia subrostrata*. Each point represents the mean value of three determinations. ● = basal (n = 33), ▲ = 10 μM/l dopamine (n = 24), and ■ = 10 μM/l serotonin (n = 32) AC activities with the lines fit by linear regressions.

dogenous Ca linear regression was calculated to have a slope of −130 pmol cAMP (mg protein · 5 min)$^{-1}$/(mM Ca/l) while slopes of both 5-HT and dopamine stimulated activities were higher (−763 ± 75 and −463 ± 19 [pmol cAMP (mg protein · 5 min)$^{-1}$]/(mM Ca/l), respectively). The effect of calcium on the monoamine stimulated activities was significantly different ($P < 0.01$) from basal; the most pronounced effect was produced by 5-HT.

Addition of exogenous calcium ($CaCl_2$) inhibited purified pellet AC (Fig. 4). The effect of exogenous calcium was primarily on the monoamine stimulated AC activities. Monoamine stimulated AC activities were significantly inhibited ($P < 0.01$). Normally, 5-HT and dopamine stimulated AC activities were 4.8 and 3.2 times above basal, respectively; in the presence of $CaCl_2$, monoamine stimulated AC activity (above $CaCl_2$ basal) was only 3.5 and 2.0, respectively. Basal AC activity also was reduced by the addition of $CaCl_2$. The addition of exogenous calcium (50% basal AC inhibition at a concentration 2.5 mM/l) was not as effective as the endogenous calcium (about 0.25 mM/l calcium).

The phosphodiesterase and non-specific phosphatase activities of the crude homogenate pellet and the supernatant were studied to determine whether these enzyme

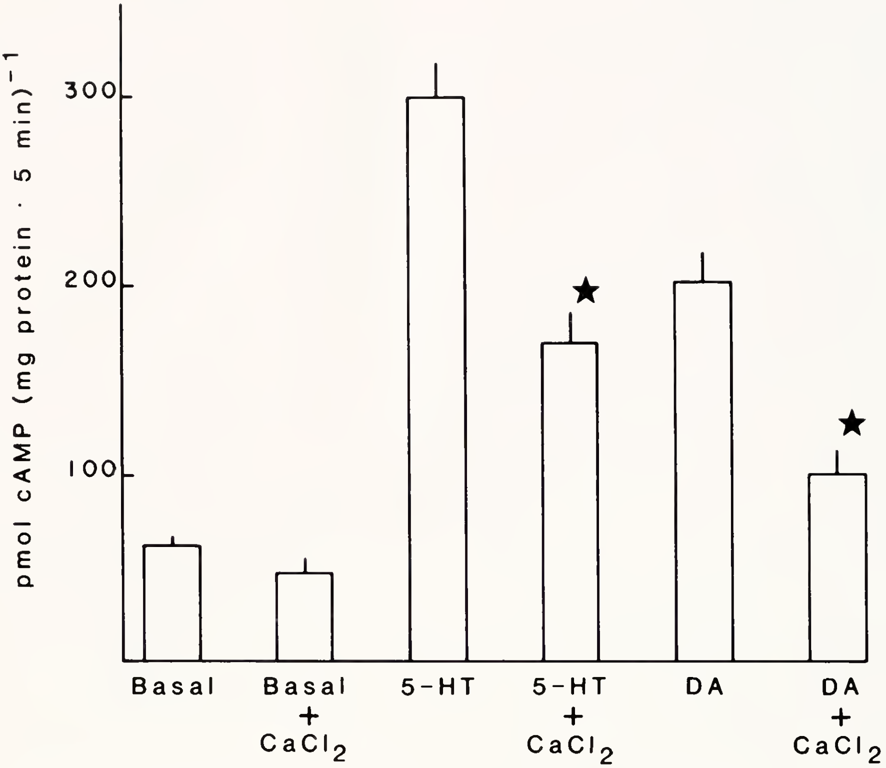

FIGURE 4. *Ligumia subrostrata* gill AC activity of basal, 10 μM/l 5-HT, and 10 μM/l dopamine with or without 2.5 mM/l $CaCl_2$. Each bar represents the mean of four gills, each assayed in triplicate using purified pellet. The vertical lines indicate the mean standard error. ★ = significantly inhibited from preparations without added Ca, $P < 0.01$.

activities could be correlated with the variability exhibited by adenylate cyclase in *Ligumia subrostrata* gills (Fig. 5). Phosphodiesterase and phosphatase activities were similar within pellet but differed in the supernatant fractions, however the activities of the two fractions were significantly different ($P < 0.001$) from each other. Supernatant phosphatase activity was 4.5 times higher than that observed in the pellet, whereas phosphodiesterase was 2.5-fold higher. The summation of both enzyme activities was 267 ± 67 pmol cAMP degraded (mg protein · 5 min)$^{-1}$ (n = 9) in the pellet fraction and a significantly higher ($P < 0.001$) 1038 ± 213 pmol cAMP degraded (mg protein · 5 min)$^{-1}$ in the supernatant fraction. Since the enzymes catabolizing cAMP were measured under optimal conditions, their activity in the pellet fraction appears to be too small to have an appreciable effect on the cAMP concentrations present in the AC activity assay. Rarely would the AC produced cAMP exceed 2 pmol in our assay conditions and sufficient theophylline was present to inhibit phosphodiesterase activity.

Prostaglandin E_2 inhibits sodium influx (Graves and Dietz, 1982; Saintsing and Dietz, 1983) and could possibly inhibit adenylate cyclase. The addition of PGE_2 did not effect basal purified pellet AC activity. In the presence of 50 ng/ml (140 μM/ml)

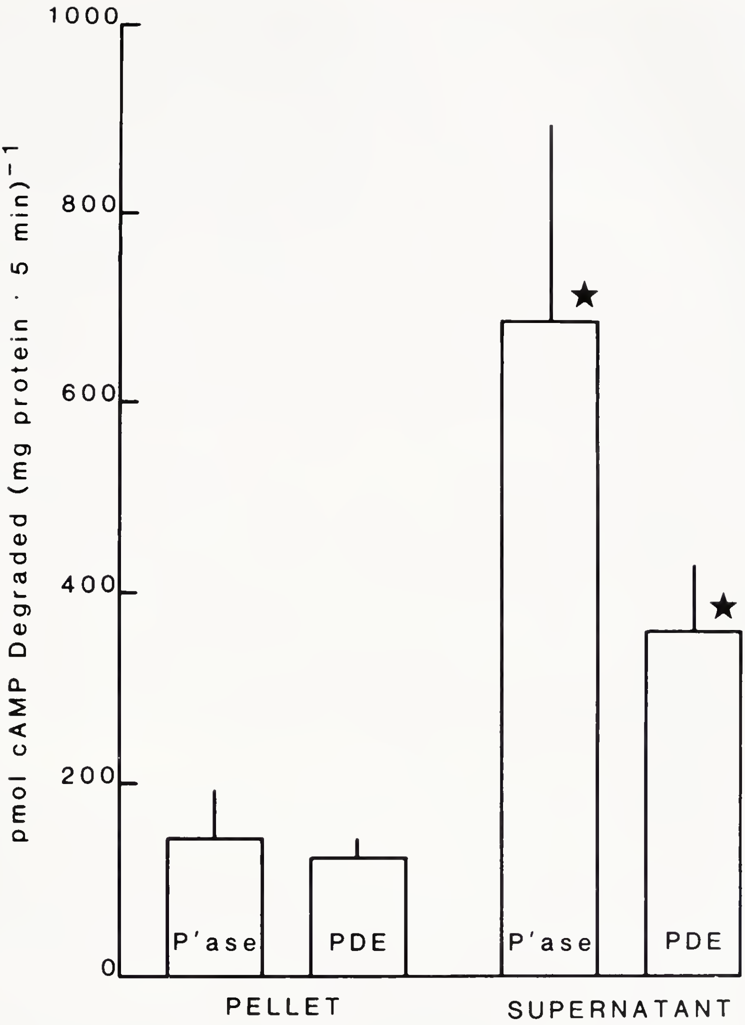

FIGURE 5. Nonspecific phosphatase and phosphodiesterase activities in the crude homogenate pellet and the supernatant fractions formed by a 3000 × *g* centrifugation of *Ligumia subrostrata* gill homogenate. Bar values represent the mean of nine gill tissues for the pellet fraction and three gill tissues for the supernatant fraction, each assayed in triplicate. Vertical lines represent the mean standard error. ★ = significantly different from the same enzyme system in the pellet, $P < 0.001$.

PGE_2, AC activity was 68.4 ± 2.7 while basal was 57.5 ± 4.0 pmol cAMP (mg protein · 5 min)$^{-1}$ (n = 2 gills each assayed in triplicate). Additionally, the inclusion of PGE_2 did not effect 5-HT or dopamine stimulated AC activity.

DISCUSSION

In freshwater mussels the sodium influx is stimulated by 5-HT and is correlated with the presence of a 5-HT stimulated adenylate cyclase. Basal flux values presented are consistent with sodium transport values previously reported (Dietz, 1979), while the 5-HT stimulated values compare with those observed in salt-depleted mussels (Murphy and Dietz, 1976; Dietz, 1978; McCorkle and Dietz, 1980; Scheide and Dietz, 1982). We have reported that the sodium influx in non-salt-depleted *L. subrostrata* and *C. texasensis* increases, when the mussels are injected with dibutyryl cAMP, 5-HT, and dopamine (Dietz *et al.,* 1982; Graves and Dietz, 1982). In addition, *L. subrostrata* gill tissue has both 5-HT- and dopamine-stimulated AC activity (Scheide and Dietz, 1983). Although both 5-HT and dopamine increase cAMP in the gill epithelium (Scheide and Dietz, unpub.), and both control gill ciliary motility (Paparo and Murphy, 1975, Paparo, pers. comm.), only serotonin stimulates sodium uptake by a cAMP-dependent transport system in isolated gills (Dietz and Graves, 1981; Dietz *et al.,* 1982). Since 5-HT stimulates Na transport and also stimulates gill AC activity in two different bivalve families (Unionidae and Corbiculidae), the serotonin-adenylate cyclase coupled sodium transport may be present in most ion regulating bivalves.

Serotonin stimulated AC activity was present in all tissues investigated in *Ligumia subrostrata.* Serotonin is a ubiquitous neurotransmitter substance in bivalves (Hiripi, 1968, 1972; Sweeney, 1968; Smith, 1982), and 5-HT is responsible for many functions including: regulation of gill sodium transport (Dietz *et al.,* 1982), control of ciliary motility (Gosselin, 1961; Paparo and Murphy, 1975), muscle relaxation (Salanki and Hiripi, 1970; Painter, 1982), rhythmic activities (Hiripi and Salanki, 1973), and heart contraction (Higgins, 1974; Painter and Greenberg, 1982).

The comparatively high 5-HT stimulated AC activity in the foot muscle, when compared to the other tissues, may be due to adaptations for the control of burrowing and locomotion. Serotonin is a neuromuscular transmitter for a number of molluscan muscles (Painter, 1982; Vitellaro-Zuccarello *et al.,* 1983).

Calcium concretions in bivalve tissues may contribute to AC activity variability in several ways. Calcium bound to the concretions can become free in solution (Istin and Girard, 1970), thus inhibiting AC activities. Istin and Girard (1970) report two pools of calcium in mantle tissue; ionized and bound. Exchange between these pools occurs and may be inhibited by acetazolamide, implying carbonic anhydrase mediation. If a system similar to that found in the mantle occurs in the gill tissue, free calcium levels will increase in the resuspended homogenate pellet since carbonic anhydrase is present in gill tissue (Henry and Saintsing, 1983).

Calcium regulates AC activity (basal, 5-HT- and dopamine-stimulated) in the *in vitro* preparation and may contribute *in vivo.* Calcium (0.1–0.5 mM/l) inhibition of AC activity has been observed before (Hynie and Sharp, 1971; Marumo and Edelman, 1971; Bockaert *et al.,* 1972; Brostrom *et al.,* 1977; Litosch *et al.,* 1982; Schmidt *et al.,* 1982). Usually, AC inhibition occurs only when calcium is added to the system, but the endogenous calcium in mussel gills appears sufficient to inhibit the activity substantially. A source of endogenous calcium present in the resuspended crude homogenate pellet is the calcium concretions that contribute at least 25% of the dry gill weight (Silverman *et al.,* 1983). If all of the calcium concretions were solubilized in our crude homogenate pellet, the calcium concentration would be >60 mM/l. Separating the calcium concretions by centrifugation and analyzing the supernatant for Ca indicates the "soluble" calcium concentration is rarely greater than 0.4 mM/l. Gill AC levels are reduced by 70% at an endogenous "soluble" Ca concentration of 0.2–0.3 mM/l, but purified pellet AC activity is inhibited 50% by 2.5 mM/l exogenous Ca. In our studies, the specific mechanism of action of Ca cannot be determined. Calcium may modulate 5-HT and dopamine action at the AC receptor level or Ca may decrease the basal AC activity due to the formation of Ca-ATP instead of Mg-ATP; the required substrate (Birnbaumer, 1973). However, we observed that the Ca effect was more pronounced on monoamine stimulated AC activity.

Calcium is typically associated with the activation of phosphodiesterase activity. Theophylline (in the concentration used in determining AC activity) inhibited 50% of the gill cAMP catabolic activity. Similar inhibition of phosphodiesterase activity was observed in *Mercenaria mercenaria* ventricle (Hess *et al.*, 1981). The remaining cAMP degrading activity was defined as phosphatase activity and should be constant in all similar preparations. In the gill homogenate pellet, the enzymatic degradation levels do not indicate that this cAMP catabolic activity significantly changes the AC activity measurements in bivalve tissue. The high level of cAMP catabolism occurring in the homogenate supernatant agrees with the previous observations that these degradative enzymes are found predominately in the soluble fraction: *e.g.*, mammalian skeletal muscle (Gain and Appleman, 1978) and cerebral cortex (Kakiuchi *et al.*, 1978). Supernatant AC activity, although greater than zero, was not significantly stimulated by 5-HT (Scheide and Dietz, 1983), perhaps masked by the substantial cAMP degradative enzymes.

Prostaglandin E_2 inhibits sodium transport and PGE_2 synthesis is inhibited by injections of serotonin or dibutyryl cAMP, thus reversing the PGE_2 effect (Saintsing *et al.*, 1983). However, crude homogenate pellet AC activity was unaffected by PGE_2 (Scheide and Dietz, 1983). Since the sodium influx stimulatory system (5-HT) and the inhibitory system (PGE_2) appear to be linked (Saintsing and Dietz, 1983), the purified pellet preparation was used to minimize the calcium interference. Addition of exogenous PGE_2 still did not significantly change AC activity from normal values; an observation similar to that reported for the rat outer medulla (Jackson *et al.*, 1980).

Sodium transport is positively modulated by a serotonin-cAMP system in freshwater bivalves. The Na regulatory system functions to maintain sodium balance during periods of ion depletion, water gain (the consequence of inhabiting a freshwater environment), or fluid loss. Fluid loss may be either through renal output or blood loss through "hemal pores" in the foot. We have noted a serotonin induced weight gain in mussels (unpub.) and the foot becomes engorged with blood. We have observed blood being expelled from the extended foot especially when the mussels are handled. Blood loss through "hemal pores" is documented in gastropods (Greenaway, 1970) and this blood loss may account for the observation that "mantle cavity fluid" is more similar to blood than pondwater (Matsushima and Kado, 1982).

Regulation of Na in freshwater mussels is a rapidly acting mechanism (initiated within 10 min) and may continue for several hours (Scheide and Dietz, 1983). The serotonin mechanism may be an integral part of the circadian rhythms exhibited by freshwater bivalves. Ion transport (Graves and Dietz, 1980; McCorkle-Shirley, 1982), as well as oxygen consumption and activity rhythms (Hiripi and Salanki, 1973; McCorkle *et al.*, 1979), exhibit coincidental phase relationships, being elevated during the dark phase and depressed during the light phase. Coordination of these activities is probably ganglionic, but at this time the site associated with monitoring sodium concentration and regulating blood Na is unknown.

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PHOTOBIOLOGY OF THE SYMBIOTIC SEA ANEMONE *ANTHOPLEURA ELEGANTISSIMA:* PHOTOSYNTHESIS, RESPIRATION, AND BEHAVIOR UNDER INTERTIDAL CONDITIONS

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ABSTRACT

Continually immersed specimens of *Anthopleura elegantissima* freshly collected from the shore receive at least 34–42% of their respiratory carbon requirement from their endosymbiotic zooxanthellae. This value decreases to 17% when intertidal sea anemones are exposed to air for 15 h during daytime spring low tides, primarily owing to a reduction in photosynthesis by the algae, which are shaded when the anemone retracts its tentacles and contracts its marginal sphincter. Contraction of continually immersed anemones during peak irradiance likewise decreases their photosynthesis. This behavior is primarily in response to high levels of intracellular oxygen generated by the symbiotic algae, although ultraviolet radiation also causes contraction, especially in aposymbiotic individuals which have lower concentrations of UV-B absorbing substances than do symbiotic anemones. Although *A. elegantissima* may vary its photosynthetic capacity according to long-term average light conditions, behavioral adaptations such as contraction and attachment of debris to the column verrucae protect the animal and its algal symbionts from deleterious photodynamic effects during short-term exposure to peak levels of irradiance.

INTRODUCTION

Anthopleura elegantissima (Brandt) is the most abundant sea anemone on the west coast of North America (Hand, 1955) and has a vertical range from 0 (mean lower low water) to more than +2 m above tidal datum (Hand, 1955; Littler *et al.,* 1983). At the latter tidal height the anemone is exposed to air approximately 90% of the time (Ricketts, 1934), frequently for several days without immersion (Fig. 1). The morphological (Francis, 1973; Shick *et al.,* 1979), reproductive (Francis, 1973; Sebens, 1980, 1981; 1982a, b), behavioral (Hart and Crowe, 1977; Shick, 1981), and physiological (Hart and Crowe, 1977; Shick, 1981) adaptations contributing to success under such physically stressful conditions have received much attention, as have the community ecological ramifications of the dominance of large areas of substrate by this anemone (Taylor and Littler, 1982).

Although it has not been stated explicitly, the nutritional contribution by this sea anemone's endosymbiotic dinoflagellates (zooxanthellae) must also be a major factor allowing a sessile suspension feeder to exist in the food-limited upper intertidal zone, and *A. elegantissima* indeed ranks among the top primary producers in its habitat, based on measurements of photosynthesis and respiration in submerged individuals

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$1\text{-}\beta$ = zooxanthella:animal biomass ratio; CZAR = contribution of zooxanthellae translocated carbon to animal daily respiration; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $\dot{n}_{O_2}$ = mass-specific rate of oxygen uptake or evolution; P_z net = net production by zooxanthellae; $_t\dot{q}$ = mass-specific rate of heat dissipation; R_a = animal respiration

(Fitt *et al.,* 1982). The ability of intertidal macroalgae to maintain photosynthesis during aerial exposure is directly correlated with their height of occurrence on the shore (Johnson *et al.,* 1974; Brinkhuis *et al.,* 1976; Quadir *et al.,* 1979; Beer and Eshel, 1983), but there have been no such studies on sea anemones. In the present paper we examine the effects of emersion on both photosynthesis and respiration in *A. elegantissima* under natural conditions of exposure and illumination.

Occupation of the intertidal zone also exposes soft bodied organisms directly to a high photon flux which has demonstrable deleterious effects on coral reef invertebrates (Jokiel, 1980; Jokiel and York, 1982; Olson, 1983). Because symbiotic individuals of *A. elegantissima* have behavioral (Pearse, 1974) and biochemical (Buchsbaum, 1968) adaptations to protect themselves against high levels of irradiance, we have extended our study to examine these defensive mechanisms and their implications for the productivity of this symbiosis.

Materials and Methods

Collection of specimens

Most specimens of *Anthopleura elegantissima* were collected on the south jetty at Bodega Harbor, California, from several clones at mean lower low water, and from a second group of clones 1.3 m above the first. These intertidal heights are the same as in our earlier study of this species (Shick, 1981); the exposure regime experienced by these anemones at the time of the various experiments (June 1980, 1982, and 1983) can be seen in the tidal curve in Figure 1. This season was chosen to minimize the difference between air and sea water temperatures while maximizing periods of intertidal exposure during daylight hours at the site.

Measurement of photosynthesis and respiration

All anemones were cleaned of adhering debris and used within 60 h (usually within 24 h) of collection. They were not fed during maintenance in the flowing sea

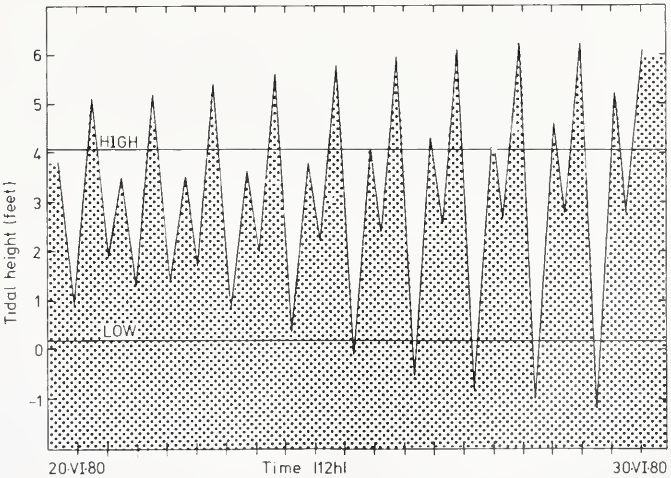

FIGURE 1. Tidal curve at Bodega Harbor, California, for 20–30 June 1980. "High" and "low" indicate intertidal heights on the south jetty at which the anemones in this study were collected.

water system at the Bodega Marine Laboratory, University of California, prior to the experiments. Photosynthesis and respiration were measured as oxygen fluxes at 15°C in air and in Millipore filtered (0.45 μm pore size) sea water in Gilson all-glass submarine respirometers, the contents of which were continuously stirred with magnetic spinbars, with at least hourly venting to the atmosphere. Aerial and aquatic measurements of oxygen fluxes under natural light conditions were synchronized to coincide with emersion and immersion of anemones at the two intertidal heights on the jetty. The temperature-stabilized respirometers, located in the courtyard of the Bodega Marine Laboratory, were situated to simulate the light and shade patterns experienced by the anemones on the jetty. All experiments were conducted on days which had fog in the morning and light overcast in the afternoon.

Chlorophyll and protein determinations

Zooxanthella:animal biomass ratios (symbolized as 1-β, following Muscatine *et al.*, 1981) of the individual sea anemones were determined by the method of McKinney (1978), with modifications. Briefly, individual anemones were weighed and then homogenized in 10 cm^3 Moore's calcium-free artificial sea water (Cavanaugh, 1975) with 0.5 mM EDTA added, in a Brinkmann Polytron homogenizer. Total protein (zooxanthellae plus animal tissue) was measured on one aliquot of the crude homogenate using the Coomassie brilliant blue technique (Bradford, 1976) after digestion in 5% NaOH. Following two 10-h extractions in 90% acetone, total chlorophyll (a and c_2) was measured according to the method of Jeffrey and Humphrey (1975). Intact zooxanthellae were separated from animal tissue in another aliquot of the homogenate by centrifugation at 900 × g in a three-step 84–21% sucrose density gradient in a Sorvall HB-4 swinging bucket rotor. The zooxanthella fraction, which accumulated at the 84–63% sucrose interface, was washed at least five times in calcium-free sea water and checked for integrity and purity under a Zeiss fluorescence microscope. The protein and chlorophyll contents of cleaned zooxanthellae were determined as for whole anemones. The zooxanthella protein:chlorophyll ratio was multiplied by the total chlorophyll content of the intact anemone, and the product was subtracted from total anemone protein, to give the animal protein content. The oxygen uptake (N_{O_2}) by living anemones could then be partitioned into zooxanthella and animal N_{O_2}, as discussed in Muscatine *et al.* (1981).

The presence of UV-B (wavelengths between 280 and 320 nm) absorbing substances in symbiotic and aposymbiotic (lacking zooxanthellae) anemones was checked according to the method of Jokiel and York (1982). Aliquots of the homogenates from the chlorophyll determinations were extracted in a 1:6 dilution with distilled water, placed in quartz cuvettes, and scanned on a calibrated Shimadzu spectrophotometer. The peak with an absorbance maximum at 320 nm was quantified in both groups of anemones.

Direct calorimetry

To confirm that intertidal specimens of *A. elegantissima* remain fully aerobic during emersion, coordinated measurements of oxygen uptake ($\dot{n}_{O_2}$, μg $O_2 \cdot h^{-1} \cdot g^{-1}$) and heat dissipation (${}_t\dot{q}$, mW $\cdot g^{-1}$) were made. Freshly collected anemones were transported to our laboratory in Maine, maintained for 4 days without feeding at their normal exposure regime in a tidal simulator, and their $\dot{n}_{O_2}$ and ${}_t\dot{q}$ measured in the dark during their normal exposure period, as described in Shick (1981). Rates of oxygen consumption were compared to rates of heat dissipation using a standard oxycaloric equivalent of 1 mg $O_2 \cdot h^{-1}$ = 3.906 mW (Gnaiger, 1983).

Contribution of zooxanthella carbon to animal respiration

Oxygen flux data [$\mu g\ O_2 \cdot (\text{mg anemone dry weight})^{-1} \cdot h^{-1}$] were converted to carbon equivalents, assuming a zooxanthella photosynthetic quotient (PQ) of 1.0 (Muscatine *et al.*, 1981) and an anemone respiratory quotient (RQ) of 1.0 (Fitt and Pardy, 1981), to enable the calculation of the contribution of translocated zooxanthella carbon to the host animal's respiratory demand (CZAR) using the equation of Muscatine *et al.* (1981). This calculation was further based on measured zooxanthella:animal protein biomass ratios of 0.026 ± 0.008 (S.D.) and 0.034 ± 0.016 in high and low shore anemones, respectively, and a zooxanthella photosynthate translocation factor of 40% (Trench, 1971). For additional assumptions of the method, see Muscatine *et al.* (1981).

Behavioral responses to illumination

Groups of 15 mid-intertidal anemones were exposed to seven experimental conditions of illumination during continuous immersion. The degree of expansion or contraction of individual anemones was noted hourly during daylight and occasionally at night for 2.5 days. Expansion state was scored as: 0—tentacles fully retracted and marginal sphincter contracted; 1—25% of oral disk exposed; 2—50% of oral disk exposed; 3—75% of oral disk exposed; 4—oral disk completely exposed and tentacles fully extended. Experimental conditions during the behavioral observations were as follows: 1) symbiotic anemones in full sunlight during daylight hours (normal); 2) symbiotic anemones in full sunlight but beneath a clear glass plate to block ultraviolet radiation (UV-blocked); 3) symbiotic anemones exposed to $1 \times 10^{-5}\ M$ DCMU, an inhibitor of photosynthesis (DCMU); 4) aposymbiotic anemones (lacking zooxanthellae) in full sunlight (Apo); 5) Apo-UV blocked; 6) symbiotic anemones in the laboratory under dim artificial light (maximum irradiance 25 $\mu\text{Einsteins} \cdot m^{-2} \cdot s^{-1}$) (Dim); and 7) Apo-Dim.

RESULTS

Rates of respiration and photosynthesis by high and low intertidal *A. elegantissima* are shown in Figure 2, as is the range of light intensity measured hourly on the several days of these experiments. Negative values for oxygen flux ($\dot{n}_{O_2}$) indicate net oxygen consumption; positive values show net oxygen production.

The coordinated measurements of $_t\dot{q}$ and $\dot{n}_{O_2}$ indicated that intertidal anemones remained fully aerobic during 15 h in air, so that measurements of oxygen uptake can be used to quantify total energy metabolism. Directly measured $_t\dot{q}$ was 1.139 $mW \cdot (g\ \text{dry weight})^{-1}$ ± 0.129 (S.D.)., and the caloric equivalent of $\dot{n}_{O_2}$ was 1.198 ± 0.054 $mW \cdot g^{-1}$ during the last 12 h in air. These mean rates are not significantly different ($t = 0.941$, df = 8, $P > .10$).

Zooxanthella:animal biomass ratios (1-β), total chlorophyll concentrations, net photosynthesis by zooxanthellae (P_z net, in carbon equivalents), animal respiration (R_a, in carbon equivalents), and CZAR are presented in Table I. Also shown are the results of *t*-tests between the means of these values in low and high shore anemones, and between means of low shore anemones and high shore anemones measured during continuous immersion. There are no significant differences between low and high shore specimens in 1-β or total chlorophyll concentration. The large reduction in CZAR in high shore relative to low shore anemones is due primarily to the reduction in P_z net in the former. Consistent but insignificant differences in P_z net and R_a between low shore anemones and high shore conspecifics measured during continuous immersion combined to produce a significantly greater CZAR in the latter.

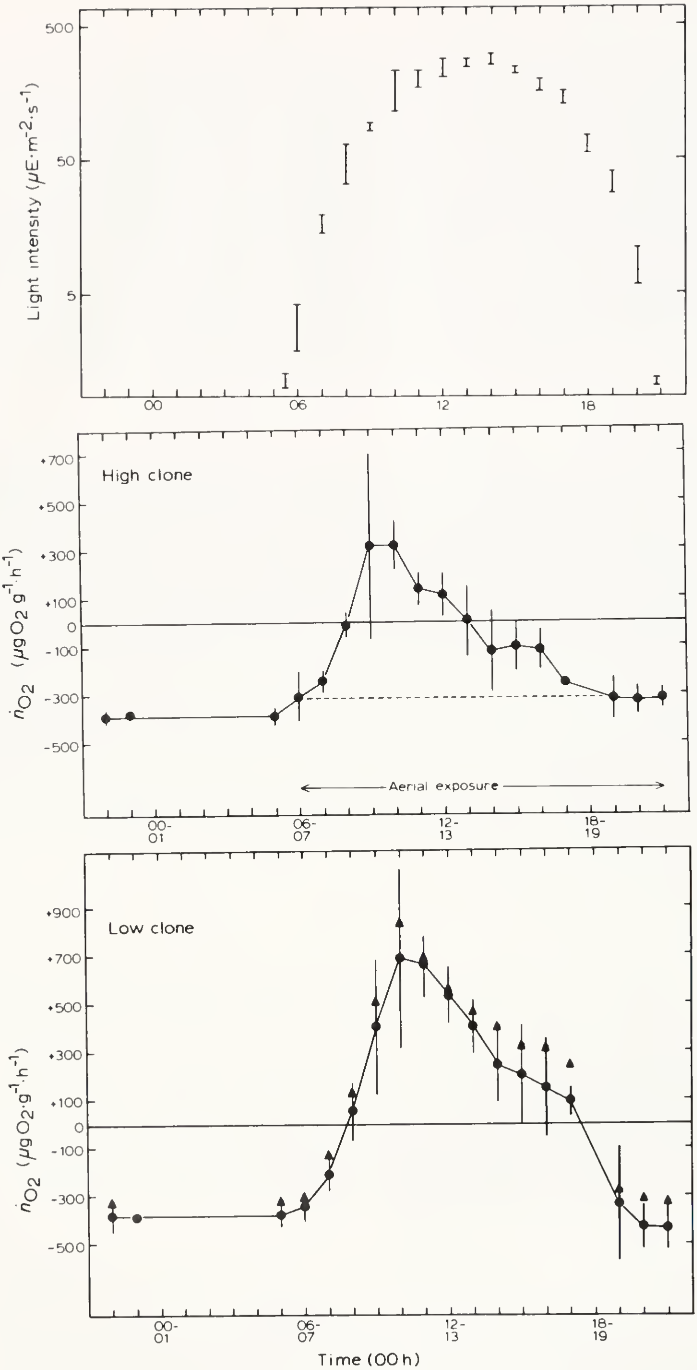

FIGURE 2. Hourly light intensity (irradiance) and oxygen flux ($\dot{n}_{O_2}$) in high and low shore specimens of *Anthopleura elegantissima* measured under natural conditions of ambient sunlight and intertidal exposure. Hours are in Pacific Daylight Time. Vertical bars indicate the range of hourly irradiance during the three days of the measurements; values for $\dot{n}_{O_2}$ are means and 95% confidence intervals. The duration of aerial exposure in the high shore anemones is indicated by the horizontal line. Also shown are the mean values (triangles) for $\dot{n}_{O_2}$ in two high shore specimens measured under low shore (continuous immersion) conditions. Oxygen flux data have not been weight adjusted, as mean weights for high and low shore anemones were the same [high: 0.549 ± 0.132 (S.D.) g dry weight; low: 0.534 ± 0.125 g dry weight)].

TABLE I

Components of organic productivity in high and low shore Anthopleura elegantissima, *and in high shore anemones under low shore (continuous immersion) conditions (High/S)*

	$1\text{-}\beta$		Chl		$P_z$net†		R_a†		CZAR†	
High	0.026 ± 0.008 (10)		0.331 ± 0.069 (10)		1.255 ± 0.046 (4)		2.853 ± 0.297 (4)		17.7 ± 1.6 (4)	
		NS		NS		**		NS		**
Low	0.034 ± 0.016 (10)		0.357 ± 0.098 (10)		2.713 ± 0.046 (4)		3.193 ± 0.260 (4)		34.0 ± 1.6 (4)	
		NS		NS		NS		NS		*
High/S	0.033 ± 0.024 (2)		0.328 ± 0.193 (2)		2.858 ± 0.047 (2)		2.748 ± 0.141 (2)		41.6 ± 1.4 (2)	

$1\text{-}\beta$ = zooxanthella:animal biomass ratio; Chl = total chlorophyll concentration ($a + c_2$, μg per mg anemone dry weight); P_z net = net production by zooxanthellae (mg C · day^{-1}); R_a = animal respiration (mg C · day^{-1}); CZAR = contribution of zooxanthellae translocated carbon to the animal's daily respiration (percent). Values are means ± S.D. (N). Results of Student's *t*-tests are expressed as: NS ($P > .05$); *($P < .01$); **($P < .001$).

† $P_z$net, R_a, and CZAR have been calculated from the data in Figure 2, where high shore anemones have a mean body dry weight of 0.549 ± 0.132 (S.D.) g, and low shore anemones weigh 0.534 ± 0.125 g.

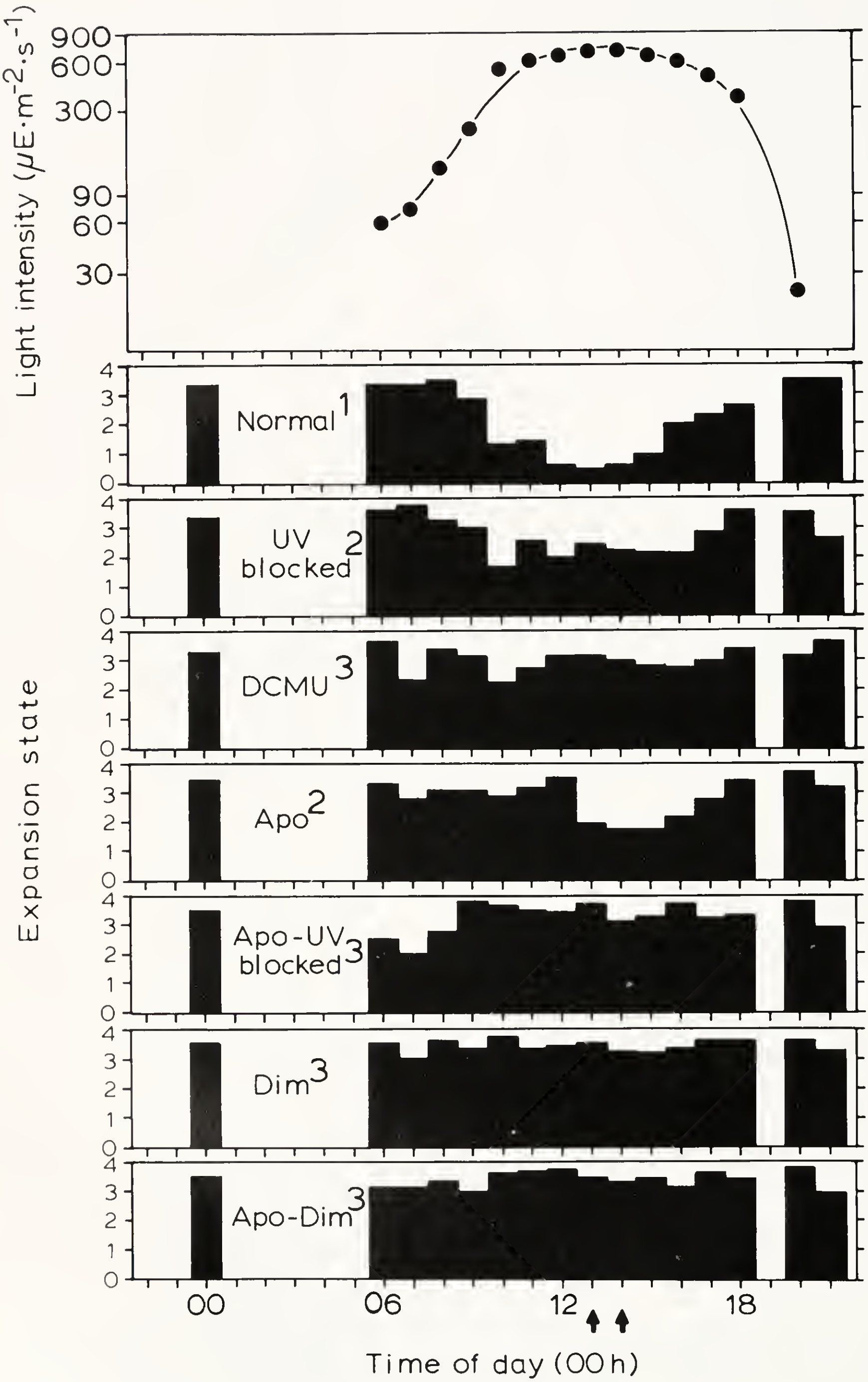

FIGURE 3. Sunlight intensity (irradiance) and mean expansion state in mid-shore *Anthopleura elegantissima* under continuous immersion. Values of light intensity are hourly means on the two days of the experiments. Bars indicate the mean expansion state of 15 anemones in each treatment group measured on two consecutive days. "Dim" and "Apo-dim" anemones were maintained in the laboratory at a maximum

The high absorbance at 320 nm by aqueous extracts of symbiotic specimens of *A. elegantissima* may indicate the presence of the S-320 pigment described by Shibata (1969), and investigated further by Jokiel and York (1982), in a variety of corals. There was no difference between low and high intertidal symbiotic anemones in their UV-B absorbance (t-test, $P > .10$), so these data were pooled. Symbiotic anemones had a higher UV-B absorbance [0.581 absorbance units per gram anemone wet weight ± 0.208 (S.D.), n = 20] than did aposymbiotic specimens (0.168 ± 0.113 absorbance units per gram, n = 10; $t = 5.295$, df = 28, $P < .001$).

Results of the behavior experiments are presented in Figure 3, as are the mean light intensities measured at the times of the observations of the anemones during the 2.5 days of the study. The degree of expansion of symbiotic anemones in natural sunlight was inversely related to light intensity above approximately 200–300 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$. When symbiotic anemones were screened from UV radiation the results were qualitatively similar, but analysis of variance and Student-Newman-Keuls multiple comparison test applied to the pooled data for the two hours of maximum irradiance (1300 and 1400 h Pacific Daylight Time) revealed that the extent of contraction was less than in symbiotic anemones not shielded from UV (Fig. 3). Inhibition of zooxanthella photosynthesis by DCMU eliminated the contraction response of symbiotic anemones to natural sunlight, but aposymbiotic anemones nevertheless did contract at midday despite their lack of zooxanthellae. Screening aposymbiotic anemones from UV eliminated this contraction, probably because these anemones had a lower concentration of UV-B absorbing substances than did symbiotic specimens. Freshly collected anemones maintained under dim illumination remained fully expanded throughout the experiment, which suggests that there is no tidal or circadian rhythm of contraction.

DISCUSSION

As in our previous studies of intertidal sea anemones (Shick *et al.*, 1979; Shick, 1981), exposure of *Anthopleura elegantissima* to air resulted in a decrease in the rate of oxygen uptake ($\dot{n}_{O_2}$) in the dark (Fig. 2). The absence of an oxygen debt (enhanced $\dot{n}_{O_2}$) in intertidally acclimatized *A. elegantissima* upon reimmersion was taken to indicate their lack of reliance on anaerobic metabolism during exposure (Shick, 1981). This has now been confirmed using coordinated measurements of heat dissipation ($_t\dot{q}$) and oxygen uptake during 15 h in air: the agreement of measured $_t\dot{q}$ with that predicted from $\dot{n}_{O_2}$ demonstrates that there is no anaerobic contribution to total metabolic heat dissipation during emersion. Although contraction in air presumably causes tissue hypoxia (and hence, diminished $\dot{n}_{O_2}$) by decreasing the surface area:mass ratio and by increasing diffusion distances in tissues (Shick *et al.*, 1979), this is offset to some extent by a continuing irrigation of the coelenteron during exposure. We have observed that intertidally-acclimatized anemones pump water out of the coelenteron, onto the exposed oral disk, and back into the coelenteron, by alternating contraction and relaxation of the column muscles.

Even more pronounced than the decrease in $\dot{n}_{O_2}$ during aerial exposure is the

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of 25 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$. See text of Materials and Methods for explanation of treatment groups and expansion state (the latter ranging from 0: fully contracted to 4: fully expanded). Arrows indicate data during the hours of peak irradiance (689 and 696 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$), which were analyzed statistically. Analysis of variance indicated significant heterogeneity among the mean expansion states (F = 70.998, df = 6, $P < .001$). Treatment groups having means not significantly different (Student-Newman-Keuls multiple comparison test) at $P = .001$ share superscripts.

decline in net oxygen production by the zooxanthellae (P_z net) (Fig. 2; Table I). This is doubtless due to the shading of the algae when the anemone retracts its tentacles and contracts its marginal sphincter, covering its oral disk. Desiccation reduces the rate of photosynthesis in intertidal macroalgae (Brinkhuis *et al.*, 1976; Beer and Eshel, 1983), but this is not a factor in the present study, as anemones were exposed to humid air in closed respirometers, and the tissue water content upon their removal from the respirometers was the same as in unexposed anemones (77%). The large variation in oxygen fluxes in anemones during daylight (Fig. 2) probably arises from individual behavioral differences, specifically, the extent to which the oral disk is covered and thus the degree of shading of the zooxanthellae.

Although *A. elegantissima* is as productive as sympatric macroalgae (Fitt *et al.*, 1982), this conclusion is based on short-term measurements of photosynthesis in submerged specimens. Because of the large decrease in P_z net due to shading during contraction in air, productivity estimates for this intertidal sea anemone must be reduced to the extent that populations are exposed to air during the tidal cycle. Such is not the case in many high intertidal macroalgae, which do not reduce photosynthesis during emersion (Johnson *et al.*, 1974; Quadir *et al.*, 1979; Beer and Eshel, 1983).

Because contraction to reduce desiccation during aerial exposure decreases zooxanthella photosynthesis more than it does animal respiration, the contribution of the zooxanthellae to the intertidal animals' respiratory carbon requirement (CZAR) is also significantly reduced when compared with the subtidal anemones (Table I). When oxygen flux in high shore anemones was measured during continuous immersion (Fig. 2), there was no significant difference in P_z net between these specimens and true low shore anemones, yet CZAR in the high shore individuals was significantly greater (Table I). This was largely due to the lower respiration rate in the high shore anemones under subtidal conditions, which may represent their "conservationist" approach to energy utilization (Newell and Branch, 1980; Shick, 1981).

Our values of 34% for CZAR in low shore *A. elegantissima* and 42% in high shore conspecifics during continuous immersion are in good agreement with that of 45% obtained by Fitt *et al.* (1982). The actual value of CZAR depends, *inter alia,* on the percentage translocation of algal photosynthate to the animal host, and both of these studies used the conservative figure of 40% based on Trench's (1971) ^{14}C-translocation experiments. These calculations of CZAR also depend on the rate of respiration by the zooxanthellae, which is not measured but rather assumed to be in direct proportion to their biomass fraction ($1\text{-}\beta$) of the entire symbiosis (Muscatine *et al.*, 1981). This assumption does not incorporate any possible scaling effects on mass-specific $\dot{n}_{O_2}$ in algal unicells and intact animal tissues, so algal respiration may be greater and P_z net lower than assumed. More recently, based on rates of carbon fixation, and zooxanthella biomass and doubling time, Muscatine *et al.* (in press) have suggested that translocation of photosynthate from algae to host may be greater than 40%, perhaps twice what was accepted previously. If true, this would double the value of CZAR and push *A. elegantissima* much closer to full photoautotrophy with respect to respiratory carbon than our results and those of Fitt *et al.* (1982) indicate. Regardless, these caveats do not alter our basic finding that intertidal exposure greatly reduces photosynthesis and hence CZAR in *A. elegantissima,* and must in turn place a premium on the efficiency of capture and utilization of prey in high shore individuals (Zamer and Shick, in prep.).

Surprisingly, net oxygen production by both low and high shore *A. elegantissima* reached its peak before maximum light intensity occurred, and oxygen production actually declined during the period of highest irradiance (Fig. 2). Observations of anemones in the respirometers indicated that the decline in photosynthesis coincided with contraction, even by immersed anemones, in very bright light. Even in air the anemones were partly expanded (oral disk exposed and coelenteron being irrigated) under cloudy-bright conditions, but they contracted in full sunlight. The greatest variability in the oxygen flux data consistently was found just as photosynthesis was reaching its maximum and before the highest irradiance occurred (Fig. 2). This may have been due to individual differences in photosensitivity and the onset of contraction during increasing light intensity. The similar transient decline in photosynthesis measured *in situ* during the brightest part of the day in shallow reef corals (Porter, 1980) and in *Tridacna squamosa* (Mangum and Johansen, 1982) may likewise reflect behavioral shading of the zooxanthellae.

Our observations of behavior are consistent with those of Pearse (1974), who noted that symbiotic individuals of *A. elegantissima* in tidepools remained fully expanded on slightly overcast days but contracted in midday sunlight on clear days. Pearse also suggested that the decline in photosynthesis under intense light was due to effects of light on maximally exposed zooxanthellae. However, photoinhibition of zooxanthellae apparently does not occur even at irradiances well above those in this study (Muscatine, 1980; Fitt *et al.*, 1982), and the observed decrease likely stems from the anemones' contraction to avoid the deleterious direct and indirect effects of light on both the animal and algal tissue. Therefore we performed a quantitative study of behavior to further examine this phenomenon.

The behavior experiments clearly showed that contraction by symbiotic anemones at midday was due both to effects of UV radiation and to oxygen produced within the anemones' tissues by their zooxanthellae (Fig. 3), with the latter having the greater effect. Notice that the "UV-blocked" anemones began to contract when the irradiance reached 200–300 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$ (Fig. 3), the same level at which net oxygen production by anemones shielded from UV in the glass respirometers began to decline (Fig. 2). Later, as irradiance decreased in late afternoon, the anemones began to re-expand, resulting in a plateau in oxygen flux.

Aposymbiotic anemones (which normally inhabit deeply shaded crevices among the boulders on the jetty) showed greater contraction in bright sunlight than did DCMU-treated symbiotic anemones (Fig. 3), despite the elimination of zooxanthella-produced O_2 as a proximal cause of contraction in both groups. Buchsbaum (1968) found that ectodermal pigments in *A. elegantissima* were produced under the influence of bright light, and the aposymbiotic anemones from shaded habitats in our present study indeed had lower concentrations of UV-B absorbing substances than did symbiotic anemones.

In addition to their demonstrated role as a barrier against desiccation (Hart and Crowe, 1977), the gravel, shell debris, and fragments of macroalgae that *A. elegantissima* attaches to the verrucae on its column may act as a sunscreen to protect exposed anemones from harmful effects of sunlight (Shick and Dykens, 1982). Our casual field observations suggested that partially shaded anemones attached less debris than did anemones experiencing longer exposure to direct sunlight, and that previously shaded anemones cleaned of all debris and placed in full sunlight expelled their zooxanthellae (Dykens and Shick, in prep.). Pearse (1974) also noted the expulsion of zooxanthellae by anemones from shaded habitats when abruptly transferred to bright sunlight.

The relatively low I_k value (the breakpoint in the photosynthesis *versus* irradiance curve, which indicates the onset of maximum photosynthesis) of 120 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$ in *A. elegantissima* (Fitt *et al.*, 1982) suggests that this symbiosis is adapted to low light availability. This may be related both to the turbidity of the water in the anemone's inshore habitat, as well as to the shading of the algae by the anemone's contraction to avoid desiccation during aerial exposure. Because total chlorophyll ($a + c_2$) shows a pronounced seasonal cycle, with higher concentrations occurring during the winter months (Dykens and Shick, in prep.), we suggest that photosynthetic capacity in *A. elegantissima* is optimized both by physiological characteristics of the zooxanthellae (low I_k) and by variations in chlorophyll concentration related to total irradiance, which is a function of daylength, microhabitat, and local weather conditions. Because it is probably energetically advantageous for the anemone to maintain the maximum capacity for photosynthesis on a long-term basis, chlorophyll concentrations may reflect average light conditions at a given season in a particular microhabitat. This in turn may subject the animal tissue to unduly high levels of O_2 and superoxide radicals (Dykens and Shick, 1982), photosensitized oxygenations, and other chlorophyll-mediated photodynamic effects (Clayton, 1977) on unusually bright days. Accordingly, adaptation to these short-term fluctuations in irradiance are behavioral, including gravel attachment (Shick and Dykens, 1982, Dykens and Shick, in prep.) and contraction, which modulate the amount of light reaching the algal symbionts.

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